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(54) Title: IDENTIFICATION OF AGENTS THAT PROTECT AGAINST INFLAMMATORY INJURY TO NEURONS

(57) Abstract

The present invention is directed to methods for identifying agents that inhibit the toxic effects of neurotoxins on neurons from plaque component activated mononuclear phagocytes. In addition, the present invention is directed to methods of identifying agents that inhibit mononuclear phagocyte-plaque component complex formation, plaque component activation of mononuclear phagocytes, and plaque component induced neurotoxicity of mononuclear phagocytes. The present invention is also directed to agents and pharmaceutical compositions obtained by the identification methods described herein. Additionally, the present invention describes methods for using tyramine compounds to inhibit the toxic effects of neurotoxins and methods to treat and diagnose neurodegenerative diseases and disorders.

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IDENTIFICATION OF AGENTS THAT PROTECT AGAINST INFLAMMATORY INJURY TO NEURONS

RELATED APPLICATIONS

This application claims priority to U.S. Application No. 08/717,551, filed September 20, 1996, and to U.S. Application No. 08/870,967, filed June 6, 1997, the disclosures of which are hereby incorporated by reference herein in their entirety.

REFERENCE TO GOVERNMENT GRANTS

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FIELD OF THE INVENTION

The present invention generally describes methods of identifying agents that inhibit the toxic effects of neurotoxins from plaque component activated mononuclear phagocytes on neurons, methods of using tyramine compounds to inhibit the toxic effects of neurotoxins, and methods to treat and diagnose neurodegenerative diseases and disorders.

BACKGROUND OF THE INVENTION

Mononuclear phagocytes are closely associated with diseases of the central nervous system. Microglia found in normal adult brain are highly ramified, quiescent cells that retract processes and become reactive during CNS injury (Rio-Hortega, 1932). Reactive microglia (activated brain mononuclear phagocytes) have been identified with Alzheimer's disease (AD) neuritic plaques (Bolsi, 1927; McGeer et al., 1987; Rogers et al., 1988; Giulian, 1992; Perlmutter et al., 1992; Giulian et al., 1995a). As a result, β amyloid (Aβ)-induced neuron damage is thought to involve inflammatory cells. In Alzheimer's disease, quantitative histopathology has determined that more than 80% of core plaques are associated with clusters of reactive microglia while fewer than 2% of diffuse Aβ deposits show such an association (Giulian et al., 1995a). These observations suggest that brain inflammatory responses may be directed specifically against the constituents of neuritic and core plaques. As the principal immune effector cells of the brain, activated microglia are capable of releasing such cytotoxic agents as proteolytic enzymes, cytokines, complement proteins, reactive oxygen intermediates, NMDA-like toxins, and nitric oxide (Thery et al., 1991; Giulian, 1992; Rogers et al., 1992; Lees, 1993, Banati, R.B., 1993).

Alzheimer's disease accounts for more than 15 million cases worldwide and is the most frequent cause of dementia in the elderly (Terry, R.D., Katzman, Bick, K.L. (eds), 1994) and is thought to involve mechanisms which destroy neurons and synaptic connections. The neuropathology of this disorder includes formation of senile plaques which contain aggregates of Aβ1-42 (Selkoe, D.J., 1991, Yankner and Mesulam, 1991; Price et al., 1992; Younkin, 1995). Senile plaques found within the gray matter of AD patients are in contact with reactive microglia and are associated with neuron damage (Terry, R.D., 1994 a and b, Masliah, E., et al., 1994, and Perlmutter, et al., 1992). Plaque components from microglial interactions with Aβ plaques tested *in vitro* were found to stimulate microglia to release a potent neurotoxic amine, thus linking reactive microgliosis with AD neuronal pathology (Giulian, et al., 1995). However, the plaque component, or components, which elicits neurotoxic responses in microglia remained elusive.

A second type of Aβ accumulation found in both AD and aged normal brain consists of diffuse plaques (discrete mesh-like structures of 70 to 100 μm diameter, visualized by silver staining, thioflavine S, or immunohistochemistry) which are not

associated with such pathological changes as dystrophic neurites or decline in cognitive function (Yamaguchi et al., 1988; Masliah et al., 1990, 1993). Finally, diffuse, amorphous deposits of Aβ, demonstrable only by immunohistochemistry, have been described in aged brain and as an early manifestation of AD-like pathology in Down's syndrome (Giaccone et al., 1989; Verga et al., 1989). Although the mechanisms which link neuritic and core plaques to dementia remain unresolved, two principal hypotheses have been advanced, first, that AD acts as a potent and direct neurotoxic agent (Yankner et al., 1990) or, second, that neuritic/core plaques elicit a cascade of cellular events which lead to neuronal pathology (Davies, 1994; Giulian et al., 1995a). Support for the first hypothesis comes from *in vitro* observations in which synthetic Aβ peptides appear toxic to enriched cultures of neurons (Pike et al., 1991; Cotman et al., 1992) or to various non-neuronal cell lines (Behl et al.,1994; Pollack et al., 1995). Support for the second hypothesis comes from evidence that neuritic/core plaques are not directly neurotoxic, as shown by the fact that neurons can be grown successfully atop Aβ peptides (Koo et al., 1993; Wujek et al., 1996), that neuritic/core plaques added directly to neurons do not cause neuron damage (Giulian et al., 1995a), and that Aβ peptides infused into the brain do not cause tissue injury (Games et al. 1992; Podlisny et al., 1992; Stephenson and Clemens., 1992).

Since the description by Bolsi (1927) of reactive microglia near plaques in AD brain, it has been uncertain whether these reactive non-neuronal cells actually contribute to the disease process or merely reflect ongoing pathology. Recently, however, it has become clear that reactive microglia surround only certain types of amyloid deposits in the brain (the neuritic and core plaques) while ignoring nearby deposits of other types, including diffuse plaques (Perlmutter et al., 1992; Giulian et al., 1995a). Such selectivity in the distribution of reactive glia suggest that specific signals within neuritic and core plaques drive brain inflammation. With the increasing recognition that reactive microglia can mediate neuronal injury through release of cytotoxic factors (Banati et al., 1993; Giulian et al., 1993a), speculation on the involvement of microglia in AD has encompassed the release of complement proteins (Rogers et al., 1988, 1992), cytokines (Meda et al., 1995; Mrak et al., 1995), NMDA-like toxins (Piani et al., 1991; Giulian et al., 1995a, b), and free radicals (Thery et al., 1991; Hensley et al., 1994).

The present invention demonstrates that Aβ1-42 is the plaque-derived component which elicits neurotoxic responses in microglia. Importantly, the N-terminus of human Aβ provides an anchoring site necessary for initiating this neurotoxic cascade. More particularly, the HHQK-containing sequence is found to be significant in initiation. Because HHQK-like agents suppress toxic microgliosis in AD brain, neuronal loss and dementia may thereby be slowed. The present invention provides strategies for inhibiting the action of microglial neurotoxins, thus making possible the treatment of and identification of therapies for inflammatory injury to neurons in neurodegenerative diseases and disorders such as Alzheimer's Disease, stroke, trauma, multiple sclerosis (MS), Parkinson's disease, HIV infection of the central nervous system, AIDS dementia, amyotrophic lateral sclerosis (ALS or Lou Gehrig's disease), hereditary hemorrhage with amyloidosis-Dutch type, cerebral amyloid angiopathy, cerebral amyloid angiopathy, Down's syndrome, spongiform encephalopathy, Creutzfeld-Jakob disease, and the like.

SUMMARY OF THE INVENTION

The present invention is directed to methods for identifying agents that inhibit the effects of neurotoxins on neurons from plaque component activated mononuclear phagocytes. The methods comprise comparing measured mononuclear phagocyte-plaque component complex formation in the presence of agents suspected of inhibiting complex formation to measured controls, wherein reduction of mononuclear phagocyte-plaque component complex formation compared to the controls results in detection of an agent that inhibits mononuclear phagocyte-plaque component complex formation. The methods also comprise isolating mononuclear phagocytes from an inhibited mononuclear phagocyte-plaque component complex formation, and comparing the plaque component activation of a mononuclear phagocyte from an inhibited complex in the presence of agents suspected of inhibiting activation to a measured control, wherein reduction of plaque component activation of the mononuclear phagocyte compared to the control results in detection of an agent that inhibits plaque component activation of mononuclear phagocytes. The methods may further comprise isolating a mononuclear phagocyte which is plaque component activation inhibited, and comparing plaque component induced neurotoxicity of an activation inhibited mononuclear phagocyte in the presence of an agent suspected of inhibiting neurotoxicity to a measured control, wherein

reduction of plaque component induced neurotoxicity compared to a control results in detection of an agent that inhibits a plaque component induced neurotoxic mononuclear phagocyte. The methods may also comprise isolating a plaque component induced neurotoxic mononuclear phagocyte, and measuring neuron function of a neuron in the presence of a neurotoxin from a plaque component activated mononuclear phagocyte or from a plaque component induced neurotoxic mononuclear phagocyte and an agent suspected of inhibiting the effect, wherein an increase in neuron function compared to a control results in detection of an agent that inhibits the effect of a neurotoxin from a plaque component activated mononuclear phagocyte on a neuron.

The present invention is also directed to methods of identifying agents that inhibit mononuclear phagocyte-plaque component complex formation by comparing mononuclear phagocyte-plaque component complex formation in the presence of agents suspected of inhibiting the complex formation with a control such that a decrease in complex formation as compared to the control results in the detection of agents that inhibit mononuclear phagocyte-plaque component complex formation.

In addition, methods of identifying agents that inhibit mononuclear phagocyte activation by a plaque component are embodied by the present invention and comprise comparing mononuclear phagocyte activation by a plaque component in the presence of agents suspected of inhibiting the activation to a control such that a decrease in activation as compared to the control results in the detection of agents that inhibit mononuclear phagocyte activation by a plaque component.

Other methods of the present invention are directed to identifying agents that inhibit neurotoxicity of a mononuclear phagocyte which neurotoxicity is induced by a plaque component comprising comparing such neurotoxicity of a mononuclear phagocyte in the presence of agents suspected of inhibiting neurotoxicity with a control where a decrease in mononuclear phagocyte neurotoxicity compared to the control results in the detection of agents that inhibit mononuclear phagocyte neurotoxicity induced by a plaque component.

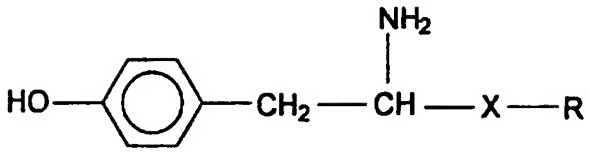
The present invention is also directed to methods of identifying agents that inhibit the effects of a neurotoxin on a neuron, which neurotoxin is from a plaque component-activated mononuclear phagocyte comprising comparing neuron function of a neuron in the presence of a neurotoxin from a plaque component-activated mononuclear

phagocyte and an agent suspected of inhibiting such effect to a measured control such that an increase in neuron function compared to said control results in detection of an agent that inhibits the effect of a neurotoxin from a plaque component activated a mononuclear phagocyte on neurons.

The present invention is also directed to methods of measuring mononuclear phagocyte-plaque component complex formation, mononuclear phagocyte activation by a plaque component, mononuclear phagocyte neurotoxicity induced by a plaque component and the effects of a neurotoxin from a plaque component activated mononuclear phagocyte on a neuron comprising labeling one of the above-identified elements followed by imaging and amplifying nucleic acids from the mononuclear phagocyte or neuron and observing the amplified nucleic acids. Further to the forgoing detection methods, plaque suppressors may be measured by observing altered mononuclear phagocyte morphology, observing the expression of cell surface molecules on mononuclear phagocytes, and observing the release of nitric oxide, free radicals, cytokines, lipoproteins, enzymes, and proteins from mononuclear phagocytes. Furthermore, inactivators of neurotoxic mononuclear phagocytes may be detected by observing a loss of metabolic function, release of intracellular material, penetration of impermeant dyes, and reduction of cell number of neurons. Yet additional methods of detecting neurotoxic blockers include observing disruption of normal cell metabolism such as metabolism of glucose, the production of ATP, maintenance of ion gradients across a cell membrane, protein synthesis, nucleic acid synthesis, and mitochondrial respiration.

Agents are also provided by the present invention which are obtained by the processes of identifying agents suspected of inhibiting mononuclear phagocyte-plaque component complex formation, neurotoxicity of mononuclear phagocytes, and effects of neurotoxins on neurons. Agents having the sequence HHQK, chloroquine, tyramine, tyramine compounds or agents having activity similar to these agents, are objects of the present invention. Pharmaceutical compositions comprising such agents are also provided for by the present invention.

Additionally, the present invention describes methods of inhibiting the toxic effects of neurotoxins in a patient comprising administering to the patient a tyramine compound or a compound that inhibits the toxic effects of neurotoxins. Preferably, the tyramine compound is a compound of the formula (I):



(I)

wherein X is an ester group, an amide group, an ether group, an alkyl group having from 1 to about 20 carbon atoms or an alkyl halide group having from 1 to about 20 carbon atoms; and R is a linear, branched or cyclic, saturated or unsaturated hydrocarbon group having from 3 to about 50 carbon atoms. In another embodiment, the present invention describes methods of treating neurodegenerative diseases or disorders in a patient comprising administering to the patient a tyramine compound or a compound that inhibits the toxic effects of neurotoxins. Another embodiment of the invention describes methods for assaying neurotoxins in a patient to diagnose a neurodegenerative disease or disorder or to monitor treatments for a neurodegenerative disease or disorder.

These and other aspects of the present invention will become apparent from the following detailed description.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 displays the chemical structure of NTox, a neurotoxin released by microglia and macrophages after exposure to senile plaques *in vitro* or *in vivo*. Chemical and enzymatic modifications of the isolated toxin have identified within NTox a phenolic hydroxyl group sensitive to tyrosinase, a ring structure sensitive to reduction by rhodium, and a terminal amine sensitive to fluorescamine (fluram) or plasma amine oxidase (PAO).

Figures 2A and B display steps in the isolation of NTox from frozen Alzheimer's diseased brain gray matter that involved extractions into ethyl acetate, acid hydrolysis and sequential gradient reverse phase high performance liquid chromatography (RP-HPLC). **Figure 2A** shows the final step of purification by RP-HPLC, using a C18 column and an acetonitrile gradient, shows a peak with elution at about 14% acetonitrile. Importantly, this peak is found in Alzheimer's diseased brains but not in control brains and corresponds to activity which is highly toxic to ciliary neurons. **Figure 2B** displays the degree of purification of neurotoxin from Alzheimer's diseased brain tissue. Dose response curves show that the ED_{50} = 10 μM in the ultrafiltrate compared with 100 pM for

highly purified toxin following acid hydrolysis and C18 RP-HPLC. From such preparations, estimations of >100,000 fold purification of toxin from human brain. The phenolic content is estimated by UV_{max} at 265 nm with a similar result obtained when values are normalized to amine content measured by fluorescamine.

Figure 3 shows the correlation between microglial clusters found in Alzheimer's diseased brains and levels of extracted neurotoxins. NTox was isolated from tissue blocks by aqueous extraction and 2-step ion exchange chromatography (DOWEX and SP-SEPHADEX) while neighboring portions of adjacent tissue stained for HLA-DR(+) microglial clusters (scored as mean number of clusters per mm^2 in 50 random field. Spearman rank correlation was highly significant (n=71 tissue regions from 6 brains; $r_s<0.0005$) suggesting that significant amounts of NTox are found in Alzheimer's diseased brains within brain structures laden with reactive microglia.

Figures 4A and B set forth the results of neurotoxin infused directly into rat brain kills neurons in vivo. Nissl stained rat hippocampus (CA3 region) 5 days after stereotaxic injection of neurotoxin. Dead and dying, pyknotic neurons are readily apparent as darkly stained, shrunken profiles in the side injected with a neurotoxin recovered from an Alzheimer's diseased brain (**Figure 4B**; Bar = 40 micron), compared to the contralateral hippocampus injected with an identical non-toxic fraction from age matched normal brain (**Figure 4A**). The inventor estimates about 100 pmoles of purified neurotoxin were contained in the 1.0 µl fluid volume injected into the hippocampus.

Figure 5 shows the specificity of Aβ1-42 to macrophages is seen by comparison with incubating either macrophages or kidney cells with microspheres coupled to Aβ1-42 for 4 hours at 37°C in the presence of increasing amounts of Aβ10-16 mixed with the culture media. As shown, competition occurs with the macrophages in a dose dependent manner while no changes in binding are seen for kidney cells. These and similar data indicate a specificity for Aβ binding to in microglia, macrophages, and other classes of microglia-like cells.

Figures 6A and B show twenty four hour exposure of human embryonic kidney (HEK) cells to 1 nM of NTox resulted in significant cell death as measured by trypan blue staining but only in those cells expressing heteromeric NMDA receptors (**Figure 6A**). Photomicrograph of trypan blue(+) control HEK cells exposed to NTox. Few blue, dead cells are noted. **Figure 6B** shows HEK cells expressing NMDA1b/2A were

also exposed to NTox for 24 hours. As seen, far larger number of dying cells appear. This NTox killing effect was found in heteromeric expression (R1/R2) and could be blocked by MK-801.

Figures 7A, B, and C show $Spheres_{A\beta 1-42}$ *in vivo*. Weeks after implantation of large microspheres (250 micron diameter) remain embedded within brain neocortex (**Figure 7A**). **Figure 7B** shows an implanted $Sphere_{BSA}$ with very few scavenger receptor(+) microglia abutting the control microsphere. In contrast, $Spheres_{A\beta 1-42}$ chronically stimulate the presence of reactive cells (**Figure 7C**). Microglia were visualized by uptake of fluorescent labeled acetylated LDL, DiI-ac-LDL Bar=40 μm, **Figure 7A**; 25 μm **Figure 7B and C**.

Figures 8A and B show scavenger receptor II mRNA in tissue surrounding sphere implants. **Figure 8A** reveals that at two weeks after implantation, there is a 5-fold increase in receptor mRNA surrounding the $Sphere_{A\beta 1-42}$ when compared to undamaged control tissue or $Sphere_{BSA}$. **Figure 8B,** in contrast, reveals that all sites had similar levels of the marker mRNA G3PDH. Data support histological changes.

Figures 9A, B, and C show infusion of Aβ1-42 into the neocortex of adult rat produces an inflammatory response 5 days later at the site of injection as seen by the presence of reactive microglia and macrophages labeled with DiI-ac-LDL (0.5 nmoles injected. **Figure 9B** reveals that co-infusion of 0.5 nmoles of Aβ1-42 plus 1.0 nmole of Aβ13-16 blocks the interaction of Aβ1-42 with microglia *in vivo* and reduces the local brain inflammatory response while co-infusion with 1.0 nmole Aβ1-5 did not alter inflammation (**Figure 9C**, Bar = 30 microns).

Figure 10 shows *in vitro* identification of drugs which inactivate microglia stimulated by Aβ1-42. Test concentrations of immunosuppressive drugs (0.1 to 10 μM) showed that only chloroquine had a protective effect and prevented appearance of neurotoxic microglia when mixed with Aβ peptides. Such in vitro assays permit rapid identification of drugs with therapeutic potential for Alzheimer's disease.

Figure 11 shows i*n vitro* identification of drugs which inactivate microglia stimulated by Aβ1-42. Test concentrations of signal transduction inhibitors (0.01 to 100 μM) showed that only compounds that block the tyrosine kinases (damacanthal and genistein) chloroquine had a protective effect and prevented appearance of neurotoxic microglia when mixed with Aβ peptides. Such *in vitro* assays permit rapid identification of

drugs which serve as lead compounds for development of therapeutics for Alzheimer's disease.

Figure 12 shows a comparison of NTox with other brain-derived compounds which contain a phenolic and terminal amine group. Tyramine appears to have significant structural similarity with NTox. Tyramine, however, has no known neurotoxic or neuroprotective properties.

Figure 13 reveals neuroprotective effects of NTox-like compounds. Test conditions include microglia stimulated with Aβ1-42, isolated NTox applied to neurons directly, or neurons mixed with 100 μM of the toxin quinolinic acid (QUIN). As shown, only tyramine prevented neuronal injury. Importantly, this protective effect did not occur with quinolinic acid which points to existence of families of molecules which could prevent microglia-mediated neuron injury.

Figures 14A-D display neurotoxic microglia activated by β-amyloid peptide. **Figure 14A** shows a fluorescence photomicrograph of neurons immuno-stained with anti-neurofilament and anti-MAP 2 antibodies found in control hippocampal cultures (1,200 cells per mm^2) that were supplemented with microglia (500 per mm^2). **Figure 14B** shows a culture identical to Figure 13A exposed to synthetic human Aβ1-42 (1 μmole/l) for 72 hours resulting in a dramatic loss of neurons (Bar = 20 microns). **Figure 14C** shows testing of various Aβ peptides in a neurotoxicity assay using rat hippocampal cultures supplemented with microglia resulting in 70-80% killing of neurons after exposure for 72 hours to human Aβ1-40, Aβ1-42, or Aβ1-42 coupled to microspheres (Spheres Aβ1-42) while elimination of microglia from the cultures prevented neuron death. The pattern of neuron killing by synthetic peptides was similar to that elicited by either isolated AD plaques or native Aβ purified from plaques. Interestingly, rodent Aβ1-40 (Arg5, Phel0, and Argl3) did not activate microglia. The Aβ peptides containing either the N-terminus of the peptide (Aβ1-11, Aβ1-16, and Aβ1-28) or C-terminus (Aβ17-43) alone also were inactive. **Figure 14D** shows the capacity of Aβ1-42 (1 μmole/l) to activate microglia examined after modification of the N-terminal region by chemical or enzymatic methods. Altering residues in the 13 to 16 domain blocked the Aβ1-42 induction of neurotoxic microglia. Cyclohexanedione (CHD) - modification of Arg5; tetranitromethane (TNM) - modification of Tyr10; diethylpyrocarbonate (DEPC) - modification of His6, Hisl3, Hisl4 with hydroxylamine used to reverse the DEPC effect;

transglutaminase (TNG) modification of Gln15; ethyl acetimidate (EAM) - modification of Lysl6.

Figures 15A-D depict inhibition of Aβ binding to microglia. **Figure 15A** shows Aβ1-42 coupled to fluorescent microspheres and the Spheres Aβl-42 monitored for binding to microglia after 4 hours at 37°C in the presence of peptides (all at 10 μmoles/1). Only peptides containing residues 13-16 were able to competitively block sphere binding. **Figure 15B** shows that enzymatic treatments of microglia altered Aβ binding to cells. $Spheres_{mal\text{-}BSA}$ (which bind to scavenger receptors) or $Spheres_{A\beta 1\text{-}42}$ were incubated with microglia for 4 hours following pre-treatment of cells with trypsin (5000 units/ml at 37°C for 60 min followed by inactivation with soybean trypsin inhibitor), with heparinase (heparin lyase EC 4.2.2.7; two consecutive treatments each of 0.01 units/ml for 60 min), or with chondroitinase ABC (chondroitin ABC lyase EC 4.3.3.4; two consecutive treatments each of 0.02 units/ml for 60 min). Binding by either $Spheres_{A\beta 1\text{-}42}$ or $Spheres_{mal\text{-}BSA}$ to microglia were reduced by trypsin. Heparinase, however, only decreased $Spheres_{A\beta 1\text{-}42}$ while chondroitinase affected neither Aβ or scavenger ligand binding sites. **Figure 15C** shows that competition with ligands again suggest the involvement of a heparin sulfate-containing site on microglia with reduction of binding in the presence of heparin sulfate (50 μg/ml) or Aβ1-16 (10 μmole/1). In contrast, scavenger receptor binding of $Spheres_{mal\text{-}BSA}$ was blocked by known scavenger receptor ligands such as dextran sulfate (500 μg/ml) or acetylated LDL (ac-LDL, 200 μg/ml). **Figure 15D** shows that plaque induction of neurotoxicity in microglia involves heparin sulfate-containing site. Microglia mixed with hippocampal neurons were treated with combinations of β-D-xyloside (1 mm), heparinase (0.02 units/ml), or chondroitinase (0.04 units/ml) and then exposed to plaques. Enzyme treatments alone, particularly that of heparinase brought on some reduction in neurotoxic activity; however, a combination of both enzymatic degradation of heparin sulfate plus competitive blockade of glycosylation by β-D-xyloside completely eliminated plaque activation.

Figures 16A-C display neurotoxic microglia blocked by Aβ peptides. **Figure 16A** shows both Aβ1-42 (1 μmoles/1) in solution and or $Spheres_{A\beta 1\text{-}42}$ (250,000 per well) added to hippocampal cultures supplemented with microglia in the presence of various synthetic Aβ peptides (all at 10 μmoles/1). Peptides containing residues 13 to 16 prevented Aβ induction of neurotoxic microglia. **Figure 16B** shows that dose curves

show a greater blocking capacity for those peptides containing residues within the 1-16 hydrophilic portion of Aβ. Addition of more hydrophobic segments (beyond residue 16) diminish the ability of peptide to block Aβ1-42 interactions with microglia. **Figure 16C** sets forth comparisons of various peptides confirm that the HHQK domain of Aβ blocks plaque activation of neurotoxic microglia.

Figure 17 sets forth a table of the effects of β-Amyloid peptides upon microglia. All peptides which contain the unmodified region encompassing residues 13-16 (shaded) block Aβ1-42 to bind to $Spheres_{Aβ1-42}$, the ability of Aβ1-42 to induce microglial neurotoxicity, and the ability of AD plaques to induce microglial neurotoxicity. NA = not applied in this neurotoxicity test, since the free peptide induces microglial toxicity.

Figures 18A-G show selective elimination of microglia from mixed hippocampal cultures. Control cultures (**Figure 18A, 18C, 18E**) show complex neuronal networks revealed by MAP-2/neurofilament immunostaining (**Figure 18A**), the presence of DiI-ac-LDL(+) microglia (**Figure 18B**), and near confluent feeder layer of GFAP(+) astrocytes (**Figure 18C**). After treatment of cultures with saporin coupled to acetylated LDL (**Figure 18B, 18D, 18F**), there was an elimination of microglia (**Figure 18D**) without effect on survival of either neurons (**Figure 18B**) or astroglia (**Figure 18F**). Bar = 25 μm. **Figure 18G** shows counts of specific cell populations with and without Sap-ac-LDL treatment confirm the specific depletion of microglia. Data are expressed as mean values +/- standard error obtained from 9 randomly selected fields from at least 5 independent cultures viewed at 200x magnification.

Figures 19A-D display constituents of solubilized native senile plaques elicit neuron killing. **Figure 19A** shows neuritic/core or diffuse plaques were isolated from cortical gray matter, solubilized in formic acid, and dialyzed against a betaine buffer. Equal amounts of plaque protein (normalized to total amine content at 400 μmoles/1) were added to neuronal cultures in the presence (100,000 cells per culture) or absence of rat microglia. As shown, solubilized neuritic/core plaque proteins (Neuritic/Core Plaque) lead to significant killing of neurons, but only in the presence of microglia. Neither solubilized diffuse plaque proteins (Diffuse Plaque) nor the betaine buffer (Buffer Control) elicited neurotoxic activity. **Figure 19B** shows size-exclusion chromatography of neuritic/core plaque proteins using two Superose 12 columns in tandem (300 mm x 10 mm

x 2; beads 10 μm diameter). The chromatogram was developed with 80% glass distilled formic acid at a flow rate of 0.3 ml per minute and monitored at 280 nm. The approximate molecular masses of the fractions were: S1, 200 kDa; S2, 45 kDa; S3, 15 kDa; S4, 10 kDa; and S5, 5 kDa. **Figure 19C** shows a histogram in which exposure to peaks S3, S4, and S5 all elicited significant increases in the percent of reactive microglia as defined by morphologic criteria, whereas peaks S1 and S2 do not. **Figure 19D** shows fractions of solubilized neuritic/core plaques applied to hippocampal cultures in the presence or absence of microglia. No neuron killing was detected in cultures free of microglia. Neuron loss appeared, however, in microglia containing cultures exposed to peaks S3, S4, and S5, all which contain Aβ.

Figures 20A-D display soluble fractions of native plaques induce microglial reactivity. Bright field photomicrographs of rat microglia cultures exposed to peak S1 (**Figure 20A**) or peak S5 (**Figure 20B**) and immuno-stained for the presence of Aβ. As shown, aggregates of Aβ are found throughout the cultures incubated with peak S5 (Bar = 25 microns). Phase photomicrographs show cultured microglia as process bearing cells with spinous surfaces typical of non-reactive cells despite exposure to peak S4 (**Figure 20C**). In contrast, microglia exposed to peak S5 retract processes and take on a reactive cell morphology similar to that found in AD brain (**Figure 20D**; Bar = 5 microns).

Figures 21A-E display toxic actions of synthetic Aβ peptides upon neurons. **Figures 21A and 21B** show high concentrations of most Aβ peptides placed in hippocampal cultures containing neurons and astroglia (but depleted of microglia) show little effect. There is, however, a generalized cytotoxic action by Aβ25-35 at >30 μmoles/l on both neurons (**Figure 21A**) and astroglia (**Figure 21B**). In the absence of microglia, none of the Aβ peptides (at 1 μmole/l) produce destruction of neurons. When rat microglia are added to neuronal cultures, however, only Aβ1-40 and Aβ1-42 elicit neuron killing (**Figure 21C**). As shown in **Figure 21D**, addition of increasing numbers of microglia show a saturated neuron killing response at a density of 150 microglia per mm^2 when incubated with 1 μmole/liter Aβ1-42; microglia found within the E18 culture at the time of plating (endogenous microglia) also showed an efficient killing capacity in the presence of Aβ. These observations point to the need to deplete neuron cultures of microglia when assessing mechanisms of Aβ toxicity. Dose response curves reveal Aβ1-

42 to be the most potent microglial stimulus with an estimated ED_{50} of 10 nmoles/l compared to 80 nmoles/l for Aβ1-40 (500 microglia per mm^2 ; **Figure 21E**).

Figures 22A-F depict cellular responses upon exposure to synthetic Aβ peptides. Phase microscopy shows that cultured rat microglia undergo morphological changes with retraction of processes when exposed to 1 μmole/l Aβ1-42 (**Figure 22E**); in contrast, 1 μmole/l Aβ17-43 (**Figure 22C**) does not alter microglial morphology which appear identical to untreated cells grown under control conditions (**Figure 22A**). Fluorescence microscopy of neuron plus microglia cultures showed robust NF(+) MAP2(+) hippocampal neurons (**Figure 22B**) that are undamaged after addition of conditioned media (10% vol/vol) from microglia incubated with 1 μmole/l Aβ17-43 (**Figure 22D**). Significant neuron loss occurred, however, if hippocampal cultures were exposed to conditioned media from microglia incubated with 1 μmole/l Aβ1-42 (**Figure 22F**). Bar = 25 microns.

Figures 23A-E display Aβ activation of microglia after coupling to microspheres. Fluorescently labeled microspheres were covalently coupled to Aβ1-42 and placed in hippocampal cultures containing rat microglia (500 cells per mm^2). After 72 hours, Aβ1-42-spheres (**Figure 23A**) were localized specifically within DiI-ac-LDL(+) microglia (**Figure 23B**, co-localization noted by arrows). In contrast, Aβ17-43-microspheres (**Figure 23C**) showed no consistent association with microglia (**Figure 23D**; Bar = 20 micron). **Figure 23E**) Comparison of capacity of Aβ in solution or coupled to microspheres (bead-bound) to elicit neurotoxic microglia (250,000 microspheres per culture; 100,000 microglia per culture; 72 hour incubation). Neuronal loss was similar if Aβ peptides were in solution or bound to beads, indicating that fibril formation, or other changes in tertiary structure, were not necessary to stimulate neurotoxic microglia.

Figures 24A-H depict fluorescent photomicrographs of hippocampal cultures after exposure to Aβ1-42. **Figure 24A** shows control cultures show complex networks of NF(+), MAP-2(+) neurons. **Figure 24B** shows exposure of cultures to 100 μmoles/liter Aβ1-42 in the absence of microglia has no effect on neuron number, while (**Figure 24C**) addition of 100 nmoles/liter Aβ1-42 in the presence of rat microglia (500 cells per mm^2) destroyed nearly all neurons. **Figure 24D-G** shows immunostaining for neuron-specific enolase (NSE) is not specific to neurons in CNS cultures as shown by immunofluorescent visualization of glia in cultures of neuron-free optic nerve, including

galactocerebroside(+) oligodenroglia (**Figure 24D**) and GFAP(+) astrocytes (**Figure 24F**) which are both NSE(+) (**Figure 24E and 24G**, respectively). Bar = 10 μm. In **Figure 24H**, ciliary neuron cultures showed that Aβ1-42 is not toxic to neurons in the absence of brain glia (Aβ1-42 only) after 48 hour exposure. Conditioned media from Aβ1-42-stimulated microglia (Microglia + Aβ1-42) did, however, kill neurons, indicating that astrocytes are not necessary to the microglial neurotoxicity.

Figures 25A-E display human microglia and neuron killing. **Figure 25A** shows only Aβ-containing fractions from solubilized neuritic/core plaques [peaks S3 (54 nmole/1), S4 (220 nmole/1), and S5 (250 nmole/l)] elicit human microglia to engage in neurotoxic behaviors. **Figure 25B** shows that when tested at 1 μmole/liter concentrations, synthetic Aβ1-40 and Aβ1-42 also stimulated release of neurotoxin from human microglia, while smaller AP fragments had no effect. Despite neuron killing, there is no evidence of increased production of nitrate or nitrite by human cells stimulated with either native (**Figure 25C**) or synthetic (**Figure 25D**) AD. **Figure 25E** shows that neuron killing could be induced by human or rat microglia exposed to 1 μmole/liter of the human forms of either Aβ1-42 or Aβ1-40. The rodent form of Aβ1-40, however, was inactive, as were fragments of human Aβ, including 1-28, 12-28, and 17-43.

Figures 26A-C display drug blockade of Aβ induced neuron killing by rat and human microglia. To investigate mechanisms of cell killing, rat microglia were stimulated with 1 μmole/1 Aβ1-42 (Rat/Aβ1-42) and human cells with fraction S5 (containing 250 nmole/1 of native Aβ1-42) from solubilized neuritic/core plaques (Human/S5 Peak). **Figure 26A** shows agents that act as free radical scavengers (vitamin E, 100 μM; catalase, 25 units/ml; glutathione, 100 μM) did not block microglial killing of neurons. No protective effects were observed with the nitric oxide synthetase inhibitors L-N-5-(1-imin-oethyl)ornithine hydrochloride (L-NIO, 10 μM) or diphenyl iodonium (DPI, 300 nM), although the NMDA antagonist AP5 prevented neuron death. **Figure 26B** shows other NMDA antagonists acting at the receptor site (AP7), at the polyamine regulatory site (ifenprodil), or at the ion channel (MK801) all blocked neuron death, while the non-NMDA glutamate antagonists (GAMS, BNQX) did not. All drugs were applied at 10μM. **Figure 26C** shows isolation of neurotoxin from culture media conditioned by Aβ-stimulated rat microglia (Aβ1-42/Microglia) or from frozen AD gray matter (AD Brain) involved extractions in ethyl acetate (pH 10.5), acid hydrolysis, and sequential gradient

RP-HPLC (C18 column using a 0 to 20% acetonitrile gradient in dH_20 with 0.1% trifluoroacetic acid). Neurotoxin activities from microglial conditioned media co-purifies with that from AD brain tissue with a co-elution using RP-HPLC at about 14% acetonitrile. Neurotoxicity was not found within control brain extracts or from unstimulated microglial culture media.

Figure 27 depicts Aβ domains and interactions with microglia. **Figure 27A** shows a phase photomicrograph of rat microglial cell adhering to Sepharose bead coupled to human Aβ1-42 peptides. **Figure 27B** shows a fluorescence photomicrograph of the same bead showing adherent cell labeled by the fluorescent microglial marker DiI-ac-LDL; Bar = 20 microns. **Figure 27C** shows rat microglial adherence to Sepharose-coupled beads after six hours. Plaque proteins derived from neuritic/core plaques provided an anchoring site for microglia, as did Aβ1-42. Importantly, Aβ1-28 also promoted bead binding, while Aβ17-43 did not. Controls included beads coupled to glycine (Control glycine) and to bovine serum albumin (Control-BSA). Data shown are expressed as the numbers of adhering cells per 100 randomly selected beads +/- standard error after 6 hour incubation at 37°C.

Figures 28A-G display that the Aβ cell binding domain is required for activation of neurotoxic microglia. Fluorescent photomicrographs showing microsphere binding to enriched cultures of rat microglia (500/mm^2) after 4 hour incubation at 37°C. Coupling of Aβ peptides to fluorescent microspheres showed that Aβ 1-42 (**Figure 28A**), Aβ 12-28 (**Figure 28D**), and Aβ 10-16 (**Figure 28E**) readily bind, while peptides Aβ 17-43 (**Figure 28B**), Aβ 1-11 (**Figure 28C**), and Aβ 1-5 (**Figure 28F**) did not. Quantitations of binding pattern (**Figure 28G**) indicated that regions of the N-terminus-containing amino acid residues 10-16 were necessary for Aβ binding to microglia. Data are expressed as mean values +/- standard error when viewed at 200x magnification.

Figure 29 displays the comparison of Aβ effects upon microglia. **Figure 29A** shows dose response curves in which although Aβ10-16 is able to bind to microglia, it did not elicit neurotoxic microglia. The addition of this microglial binding domain to Aβ17-42 (which neither binds to microglia nor elicits toxicity) created a peptide, Aβ10-42, which both bound to microglia and stimulated microglia to kill neurons. **Figure 29B** shows a diagram comparing the structures and functions of synthetic peptides. The shaded area illustrates the N-terminal portion of Aβ that differs between human and rat forms and

which appears necessary for microglial adherence.

Figure 30 is a comparison of tyrosine and various tyramine compounds as neuroprotective agents against toxic microglia. **Figure 30** shows that neuron survival was markedly enhanced by tyramine compounds, such as tyrosine butyl ester and tyrosine allyl ester.

Figure 31 is a dose response curve which compares tyramine, tyrosine methyl ester and tyrosine t-butyl ester as blockers of NTox. The graph shows that tyrosine t-butyl ester was the most potent neuroprotectant tested, while tyrosine methyl ester was ineffective at the doses tested.

Figure 32 is a graph showing the specificity of tyramine compounds to NTox. The graph shows that the NMDA agonists, quinolinic acid (QUIN) and AMAA are prevented from killing neurons when mixed with NMDA receptor blockers AP5 and MK-801. Unlike the NMDA agonists, the tyramine compounds only inhibit NTox. This data shows a selective protective effect for a toxic agent released by brain inflammatory cells.

Figure 33 provides examples of tyramine compounds that were tested for their neuroprotective effects in a neurotoxicity assay using Aβ-stimulated microglia and hippocampal neurons. A "-" sign indicates that the compound was not neuroprotective. A "+" sign indicates the compound was neuroprotective. A "++" sign indicates that the compound was highly neuroprotective.

Figure 34 is a graph showing the % maximal current peak elicited by NMDA, octopamine and tyramine compounds in the presence of a given NMDA concentration. Three days after injection of NMDA R1 and R2 subunit cRNAs into Xenopus oocytes, whole cell current measurements were done to assess the expression of the channel complex in response to increasing concentrations of NMDA; this current could be blocked by specific antagonists such as MK-801 (channel site) or AP5 (ligand site). Tyrosine t-butyl ester and tyrosine benzyl ester show an inhibition of NMDA, while octopamine does not alter the current measured. The data show that one action of neuroprotective tyramine compounds is to influence NMDA currents in toxin-sensitive neurons.

Figure 35 is a graph showing that mono-iodinated tyrosine t-butyl ester and di-iodinated tyrosine t-butyl ester have neuroprotective effects against NTox released from Aβ1-42 stimulated microglia.

DETAILED DESCRIPTION OF THE INVENTION

Plaque component associated diseases are believed to be the result of a cascade of events that occur in the central nervous system. The cascade comprises four events including (1) formation of a mononuclear phagocyte-plaque component complex, (2) mononuclear phagocyte activation by a plaque component, (3) mononuclear phagocyte neurotoxicity induced by a plaque component, and (4) the toxic effect of neurotoxins on neurons, wherein the neurotoxins are released from the mononuclear phagocytes.

An agent suspected of inhibiting mononuclear phagocyte-plaque component complex formation is referred to herein as a plaque supressor. Mononuclear phagocyte inactivators are agents suspected of inhibiting mononuclear phagocytes activation by a plaque component in accordance with the present invention. Neurotoxicity of a mononuclear phagocyte by a plaque component may be inhibited by an agent referred to herein as a neurotoxic mononuclear phagocyte inactivator. A neurotoxin blocker inhibits the effect of a plaque component activated mononuclear phagocyte or the effect of a plaque component induced neurotoxic mononuclear phagocyte.

Plaque is composed of plaque components comprising peptide, protein, and non-protein constituents, including β-amyloid and fragments thereof including and not limited to Aβ1-39, Aβ1-40, Aβ1-42, α-antichymotrypsin, apolipoproteins including and not limited to apoplipoproteins A and E, glycoproteins, proteoglycans including and not limited to heparan sulfate, and proteases, found in a mammalian central nervous system as a result of a disease. The β-amyloid sequences referred to in the present invention are described by Selkoe, 1991.

The present invention reveals that plaque components interact with mononuclear phagocytes to form a mononuclear phagocyte-plaque component complex. For purposes of the present invention, interact, and variations thereof, is used synonymously with associate, combine, attach, interfere, bond, and bind. The term "interact" or variations thereof, as used herein in connection with the action of a plaque component includes a covalent bond and an ionic bond or other means of chemical or electrochemical linkage or interaction. While not intending to be bound by any particular theory of operation, it is believed that the interaction involves cell surface compounds of the mononuclear phagocyte, such as and not limited to, cell surface receptors for the plaque components identified herein.

Cells of the central nervous system are the cells inhibited by the agent of the present invention. A cell may be a central nervous system cell, such as and not limited to a mononuclear phagocyte and a neuron. Mononuclear phagocyte, as defined in the present invention, is a target cell of a plaque component and contains specific binding sites required for activation and induction of neurotoxicity. These binding sites include cell surface proteins that contain heparan sulfate. A mononuclear phagocyte is an immune cell which has a single nucleus and the ability to engulf particles, also known as phagocytosis. Mononuclear phagocytes are found in blood and body tissues, including the central nervous system and brain, and include, for example, microglia cells, monocytes, macrophages, histiocytes, dendritic cells, precursor cells of microglia, precursor cells of monocytes, precursor cells of macrophages, microglia-like cell lines, macrophage-like cell lines, or cell lines modified to express microglia-like surface molecules that are active in accordance with the above definition of mononuclear phagocyte. Throughout the disclosure the term "mononuclear phagocyte" includes microglia cells, monocytes, macrophages, histiocytes, dendritic cells, precursor cells of microglia, precursor cells of monocytes, precursor cells of macrophages, microglia-like cell lines, macrophage-like cell lines, or cell lines modified to express microglia-like surface molecules that are active in accordance with the above definition of mononuclear phagocyte. A neuron as defined in accordance with the present invention includes a neuron-like cell line, a cell modified to express a N-methyl-D-aspartate receptor which neuron exhibits neuronal activity under typical normal, non-diseased state, conditions.

Plaque component-mononuclear phagocyte complex formation initiates a process that causes the release of neurotoxins. Since the formation of Aβ complexes with mononuclear phagocytes induces mononuclear phagocytes to become neurotoxic, the blockade of the Aβ-microglia complex formation suppresses neurotoxic mononuclear phagocytes. Compounds with the ability to block such complex formations have structural features common either to a binding domain of Aβ or to the mononuclear phagocyte binding sites, such as Aβ ligand binding domain and/or sites containing heparan sulfate, for example.

Mononuclear phagocytes may be activated by a plaque component following complex formation. Activation is also referred to herein as immune activation, markers of which are any process that renders a mononuclear phagocyte more dynamic and

characterized by activities such as and not limited to increased movement, phagocytosis, alterations in morphology, and the biosynthesis, expression, production, or secretion of molecules, such as protein, associated with membranes including complement, scavengers, Aβ, and blood cell antigens, histocompatibility antigens, for example. Production of molecules includes enzymes involved in the biosynthesis of bioactive agents such as nitric oxide synthetase, superoxide dismutase, small molecules such as eicosanoids, cytokines, free radicals and nitric oxide. Release of factors includes proteases, apolipoproteins such as apolipoprotein E, and cytokines such as interleukin-1, tumor necrosis factor as well as other molecules such as NTox and hydrogen peroxide.

Mononuclear phagocyte-plaque component complex formation and mononuclear phagocyte activation induced by a plaque component may be followed by neurotoxicity of a mononuclear phagocyte induced by a plaque component. Neurotoxicity refers to a process that leads to the injury, destruction, or death of neurons, which is measured by loss of metabolic function, release of intracellular material, penetration of impermeant dyes, reduction of cell number measured by biochemical or histological methods. For example, changes in biochemical markers such as loss of neurofilaments or synaptophysin or release of lactate dehydrogenase, or other evidence of cell injury such as penetration of impermanent dyes, including fluorescent nuclear dyes and trypan blue. These and other strategies for identifying cell injury, destruction or death, or measuring neuron function, are known to one skilled in the art and are contemplated by the present invention.

The effect of a neurotoxin from a plaque component activated mononuclear phagocyte or from a plaque component induced neurotoxic mononuclear phagocyte on a neuron may take place following complex formation, activation and neurotoxicity. Neurotoxins are defined herein as substances that injure, damage, or kill a neuron while sparing other central nervous system cells such as glia, for example. Neurotoxin blockers are agents which inhibit the effects of neurotoxins that are released from plaque component activated mononulcear phagocytes. Neurotoxic compounds, including phenolic amines such as Ntox (**Figure 1**), form a complex with molecules of neurons in such a way as to disrupt neuron function and survival. The possible actions of the neurotoxic compounds on neurons, also referred to herein as neuronal damage, include inhibition or disruption of normal cell metabolism including metabolism of glucose, the

production of ATP, and maintenance of ion gradients across cell membranes including Na^+, Ca^{2+}, and K^+ ion channels, the synthesis of proteins and nucleic acids, and mitochondrial respiration.

The phenolic amine, NTox, (**Figure 1**) has been highly purified from Alzheimer's diseased brains at greater than 100,000 fold (**Figure 2**). Levels of NTox within brain regions correlate to the number of reactive microglia clusters found in the same brain regions (**Figure 3**). Importantly, NTox isolated from Alzheimer's diseased brains destroys neurons when infused into the brains of animals (**Figure 4**). The concentrations of NTox found in Alzheimer's diseased brains are very high and suggest that this particular toxic agent accounts for the neurodegeneration which occurs in Alzheimer's disease. Thus, prevention of plaque activation of microglia would suppress production of NTox and reduce or prevent the brain damage found in Alzheimer's disease.

The cascade of events may take place as a result of one plaque component binding to a mononuclear phagocyte, or more than one plaque component binding at complex formation or a series of plaque components continuing to bind during any of the cascade events.

Any agent (also referred to herein as a test compound), compound, compounds, mixture, complex, blend, combination of atoms, elements, chemicals, biological materials including and not limited to peptides, proteins, nucleic acids, and nucleotides suspected of having inhibitory activity to one of the events in the cascade may be identified or screened in accordance with the methods of the present invention. An effective amount of a mononuclear phagocyte and a plaque component is the amount of each normally resulting in an event in the cascade, but for the addition of a suspected inhibitory agent. An effective amount will be known to a skilled artisan in view of the present disclosure and will vary depending on the use of a mononuclear phagocyte, neuron, plaque component or components, and the mammalian origin of the cells. The mammals useful for *in vivo* identification in accordance with the present invention include and are not limited to primates such as and not limited to monkey, chimpanzee, and ape, rodents, such as and not limited to rat and mouse, guinea pig, dog, cat, rabbit, and pig. *In vivo* assays performed in accordance with the methods of the invention include human central nervous system tissue, such as brain. The plaque component source useful in the present invention includes plaque of a patient suspected of having a neurodegenerative disease or disorder,

including, for example, Alzheimer's disease, hereditary hemorrhage with amyloidosis-Dutch type, cerebral amyloid angiopathy, cerebral amyloid angiopathy, Down's syndrome, spongiform encephalopathy, Creutzfeld-Jakob disease, HIV infection, AIDS dementia, Parkinson's disease, multiple sclerosis, amyotrophic lateral sclerosis (ALS or Lou Gehrig's disease), stroke, or trauma.

An effective amount of time for contacting a mononuclear phagocyte with a plaque component and an agent suspected of inhibiting the mononuclear phagocyte-plaque component complex formation is the amount of time in which inhibition of complex formation is observed and includes, for example, the amount of time the complex forms under conditions in which the suspected inhibitory agent is not present.

For purposes of the present invention, inhibit, inhibition and variations thereof are used synonymously with reduce, suppress, retard, slow, and suspend. Further, agents may be identified that completely inhibit any of the events in the cascade such that the event is arrested, stopped, or blocked. In accordance with the present invention, a mononuclear phagocyte, a neurotoxin or a neuron which is inhibited is one which is unable to display the event typically seen under typical cascade conditions. Typical cascade conditions are conditions that do not include an agent suspected of inhibiting the cascade event. Accordingly, the present invention includes the identification of agents that substantially inhibit any of the cascade events. Inhibition of events in the cascade refers to less than about 1% to about 100% of the cells of a given population that are inhibited. Preferably, a cascade event is inhibited about 10%, more preferably about 20%, more preferably about 50%, even more preferably about 75%, even more preferably about 100%. By way of example, a compound inhibits the toxic effects of a neurotoxin if, in a comparative sense, neurons that are otherwise at risk of injury, destruction or death are exposed to the compound whereby a percentage of the at risk neurons do not otherwise destruct or die. Preferably, more than 10% of the at risk neurons do not otherwise destruct or die, more preferably more than 20% or more than 50%, even more preferably more than 75%, even more preferably more than 85%, still more preferably more than 95%, and most preferably 100%.

Measuring formation of a mononuclear phagocyte-plaque component complex may be achieved by any of the standard methods known in the art. Examples of the typical methods include imaging and detection of amplified nucleic acids. An initial rapid

screen of Aβ binding to microglia *in vitro*, for example is based upon the fact that specific domains of the Aβ peptide are essential for Aβ-microglia interactions. A plaque component, mononuclear phagocyte, agent, or solid support used in any of the detection methods of the present invention, may be tagged by various methods to allow detection. These methods include and are not limited to chemical radiolabeling (such as modifications with ^{125}Iodine, such as at tyr10), synthetic incorporation of radiolabels during synthesis of peptides (such as amino acid or side chain modifications containing, for example, ^{14}Carbon, ^{3}Hydrogen, or ^{35}Sulfate), coupling to solid supports which themselves are tagged (such as the use of Aβ coupled to fluorescent microspheres, peptide labeling with fluorescent tags, such as and not limited to rhodamine and fluorescein, modifications of peptides to allow for detection via ligand binding, such as and not limited to biotinylation for biotin-avidin detection methods and anti-digoxigenin antibody detection) and enzymatic methods wherein for example enzymes are coupled to calorimetric displays such as and not limited to the peroxidase detection method.

To determine if Aβ peptide selectively bound to microglial surfaces, Aβ peptides were coupled to microspheres and incubated with cultures of microglia. Microspheres coupled to Aβ1-42, but not Aβ17-43, were readily engulfed by microglia. Comparisons among various sequences with the Aβ1-42 peptide revealed that those peptides encompassing residues 10 to 16 were essential for Aβ binding to microglia. Importantly, this binding shows specificity (selective competitive against free Aβ1-42) and demonstrates cellular selectivity to microglia and other classes of mononuclear phagocytes (**Figure 5**). The microglial binding sites for Aβ include cell surface associated molecules containing trypsin-sensitive protein component and a heparan sulfatase sensitive component.

Mononuclear phagocyte-derived binding sites may be presented in an identification assay in several ways such as and not limited to culturing of cells (microglia, microglia-like cell lines, macrophages, macrophage-like cell lines, histiocytes, dendritic cells, or cell lines modified to express microglia-like surface molecules) in test chambers, adhering or coupling membranes to test chambers, or placement of specifically isolated membrane or chemical fractions from microglia or macrophages which contain the relevant Aβ binding component that (i) show a specificity of binding to plaques and/or Aβ peptides which contain the sequence of the HHQK domain, and (ii) show involvement in

complex formation. Mononuclear phagocytes may be labeled biosynthetically or by chemical or enzymatic methods to tag a mononuclear phagocyte-derived element involved in forming a complex with a plaque component. These and other strategies for labeling plaques, peptides, or cells are known to one skilled in the art and are contemplated by the present invention. Examples of assays include and are not limited to the following.

Cell associated binding assays comprise a test agent mixed with tagged plaque, Aβ, or microglia-derived binding site. Complex formation blocked by a test compound may be indicated by a reduction in recovery of tagged materials within a formed complex when compared to a control.

A tissue slice binding assay comprises mixing an agent suspected of having inhibitory activity to a cascade event with a tagged plaque component and tissue sections of normal and abnormal mammalian brain, for example, a human tissue sample from a patient with Alzheimer's disease. Blockade of complex formation by a test compound may be observed by a reduction of tagged materials, (labeled, for example with a radiolabel, fluorescent dye, or enzyme) within formed complexes when compared to a control.

In vitro neurotoxicity assays detect an inactivator of a neurotoxic mononuclear phagocyte and employ cultures of neurons, neuron-like cell lines, or cells which have been modified to express N-methyl-D-aspartate receptors (**Figure 6**). The presence of neurotoxic activity, or a measure of neuron function, will be determined by reduction in cell number, changes in biochemical markers such as loss of cell metabolic function, release of intracellular material, penetration of impermeant dyes, such as and not limited to fluorescent nuclear dyes and trypan blue (**Figure 6**), reduction of the number of neurons, loss of neurofilament or synaptophysin, release of lactate dehydrogenase, or other evidence of cell injury. These and other strategies for identifying cell neurotoxicity or measuring neuron function, which may be displayed as cell injury, are known to one skilled in the art and are contemplated by the present invention.

In vivo binding, activation, and neurotoxicity assays involve infusion of a tagged plaque component coupled to a solid support such as microspheres (**Figure 7**) into a mammalian brain. Following infusion of materials, a test compound may be co-injected into the brain intraventricularly or intracranially by cannula with delivery systems including syringes or implanted osmotic pumps. A test compound may also be delivered

systemically by intraperitoneal, intravenous, intra-arterial, nasal, or oral routes. Evidence of immune activation of the brain by a plaque component may be monitored using histological, biochemical, or molecular methods such as quantitative reverse transcriptase polymerase chain reaction method to identify cell markers of immune responses including surface proteins, enzymes, or cytokines (**Figure 8**). The production of neurotoxic activity may be assayed by histochemical (**Figure 7**) or biochemical to identify cell markers of immune responses including injury or destruction of neurons. Neurotoxicity assays may observe a loss of metabolic function, release of intracellular material, penetration of impermeant dyes, and reduction in neuronal cell numbers.

Other methods of detecting a neurotoxin blocker, or measuring neuron function, include detecting the inhibition of normal cell metabolism including the disruption of glucose metabolism, ATP production, ion gradient maintenance across cell membranes, and protein synthesis, nucleic acid synthesis, and mitochondrial respiration. Reductions in an inflammatory marker or injury to a neuron by a test compound may be compared to a control. In addition, methods of detecting amplified nucleic acids will be known to one skilled in the art in view of the present disclosure.

A number of template dependent processes are available to amplify the target sequences of interest present in a sample. One of the best known amplification methods is the polymerase chain reaction (PCR) which is described in U.S. Patent Nos. 4,683,195, 4,683,202 and 4,800,159, and in Innis *et al.*, 1990. In addition, other methods known to a skilled artisan for amplifying nucleic acids may be used in place of PCR, such as and not limited to LCR described in EPA No. 320,308, Qbeta Replicase, described in PCT Application No. PCT/US87/00880, isothermal amplification methods, described by Walker, G. T., *et al.*, 1992, transcription-based amplification systems (TAS) (Kwoh D., *et al.*, 1989, Gingeras T. R., *et al.*, PCT Application WO 88/10315, including nucleic acid sequence based amplification (NASBA) and 3SR, Davey, C., *et al.*, European Patent Application Publication No. 329,822, describe a nucleic acid amplification process involving cyclically synthesizing single-stranded RNA ("ssRNA"), ssDNA, and double-stranded DNA ("dsDNA") which may be used in accordance with the present invention. Miller. H. I., *et al.*, PCT application WO 89/06700, describe a nucleic acid sequence amplification scheme based on the hybridization of a promoter/primer sequence to a target single-stranded DNA ("ssDNA") followed by transcription of many RNA

copies of the sequence. This scheme is not cyclic; i.e. new templates are not produced from the resultant RNA transcripts. Other amplification methods include "race" described by Frohman, M. A., 1990) and "one-sided PCR" (Ohara, O., *et al.*, 1989).

Strand Displacement Amplification (SDA) is another method of carrying out isothermal amplification of nucleic acids which involves multiple rounds of strand displacement and synthesis, i.e. nick translation. A similar method, called Repair Chain Reaction (RCR) is another method of amplification which may be useful in the present invention and which involves annealing several probes throughout a region targeted for amplification, followed by a repair reaction in which only two of the four bases are present. The other two bases can be added as biotinylated derivatives for easy detection. A similar approach is used in SDA.

Mononuclear phagocyte specific nucleic acids can also be detected using a cyclic probe reaction (CPR). In CPR, a probe having 3' and 5' sequences of non-mononuclear phagocyte specific DNA and a middle sequence of mononuclear phagocyte specific RNA is hybridized to DNA which is present in a sample. Upon hybridization, the reaction is treated with RNaseH, and the products of the probe, identified as distinctive products, generate a signal which is released after digestion. The original template is annealed to another cycling probe and the reaction is repeated. Thus, CPR involves amplifying a signal generated by hybridization of a probe to an mononuclear phagocyte specific expressed nucleic acid.

Still other amplification methods described in GB Application No. 2 202 328, and in PCT Application No. PCT/US89/01025, may be used in accordance with the present invention. In the former application, "modified" primers are used in a PCR-like template and enzyme dependent synthesis. The primers may be modified by labeling with a capture moiety (e.g., biotin) and/or a detector moiety (e.g., enzyme). In the latter application, an excess of labeled probes are added to a sample. In the presence of the target sequence, the probe binds and is cleaved catalytically. After cleavage, the target sequence is released intact to be bound by excess probe. Cleavage of the labeled probe signals the presence of the target sequence. Methods based on ligation of two (or more) oligonucleotides in the presence of nucleic acids having the sequence of the resulting "di-oligonucleotide," thereby amplifying the di-oligonucleotide, may also be used in the amplification step of the present invention.

The present invention also embodies adhering a plaque component or cell to a solid support. A solid support may be selected from any solid support known to one skilled in the art, such as and not limited to, a microsphere, liposome, sepharose, sephadex, vesicle, microbubble, polymeric bead, and the like.

Agents detected by a process set forth herein for the detection of an inhibitor of a mononuclear phagocyte-plaque component complex formation were in fact found to be inhibitory to complex formation. One plaque suppressor agent is identified herein as HHQK. Agents comprising HHQK, HHQK-like agents having HHQK activity such that they are inhibitory to complex formation include agents having a secondary or tertiary structure substantially similar to HHQK. Alternatively, HHQK-like agents or activity may be measured by similar hydrophobicity, hydrophilicity, acidity, and basicicity of the agent or side chains thereof. In addition, minor variations in the measurements identified herein are considered to be substantially similar to HHQK.

Another plaque suppressor is heparan sulfate and heparan sulfate-like agents having heparan sulfate activity such that they are inhibitory to complex formation include agents having a secondary or tertiary structure substantially similar to heparan sulfate. Alternatively, heparan sulfate-like agents or activity may be measured by similar hydrophobicity, hydrophilicity, acidity, and basicicity of the agent or side chains thereof. In addition, minor variations in the measurements identified herein are considered to be substantially similar to heparan sulfate. A composition comprising heparan sulfate or a heparan sulfate-like agent is provided herein, such that the composition is provided in a pharmaceutically acceptable carrier in a therapeutically effective amount. Suitable pharmaceutical carriers are well known in the art and described, for example, in Gennaro, Alfonso, ed., Remington's Pharmaceutical Sciences, 18th Edition, 1990, Mack Publishing Co., Easton PA, a standard reference text in this field.

The particular amount of the compositions of the invention that will be administered to the mammal for any particular condition will depend on the type of illness, and other factors such as the weight and age of the patient and route of delivery.

Identification of various plaque components shows that the components containing the HHQK sequence block Aβ binding to neurons and block neuron-killing elicited by plaques or Aβ peptides. Dose response curves showed the HHQK peptide as one of the most potent plaque suppressor peptides identified. Similarly, identification of

polysaccharides indicate that heparan sulfate (and not similar compounds such as dextran sulfate or chondroitin sulfate) block Aβ binding to microglia. To confirm the ability of HHQK to activation of microglia *in vivo*, Aβ1-42 was injected into the neocortex of rats in the presence or absence of Aβ13-16. As shown in **Figure 9**, inflammation induced by Aβ1-42 in the brain was markedly reduced if Aβ13-16 were present. Thus, by a series of identification methods, two very different compounds, the peptide HHQK and polysaccharide heparan sulfate, have been identified as potential plaque suppressor agents for use as therapy for Alzheimer's disease.

Agents which inhibit plaque component induced neurotoxicity of a mononuclear phagocyte are referred to as inhibitors of neurotoxic mononuclear phagocytes. One inhibitory agent is chloroquine. Agents comprising chloroquine compounds, chloroquine derivatives and compounds having chloroquine activity such that they are inhibitory to neurotoxic mononuclear phagocytes, include agents having a secondary or tertiary structure substantially similar to chloroquine. Alternatively, chloroquine compounds or activity may be measured by similar hydrophobicity, hydrophilicity, acidity, and basicicity of the agent or side chains thereof. In addition, minor variations in the measurements identified herein are considered to be substantially similar to chloroquine. A composition comprising chloroquine, a chloroquine compound or a chloroquine derivative may be used in a similar therapeutic and pharmaceutical manner as set forth above for the plaque suppressor agents.

Once activated by plaque components, microglia (mononuclear phagocytes) release neurotoxins. Although many drugs are known to suppress the immune system outside the brain, it is not known which drugs actually have the ability to inactivate plaque-stimulated microglia within the brain and thus prevent the immune-mediated damage caused by neurodegenerative diseases or disorders, such as Alzheimer's disease. Using the assay systems noted above, it is possible to screen for therapeutic agents that inhibit neuron-killing microglia. As shown in **Figure 10**, microglia incubated with chloroquine, unlike other immune suppressants such as dexamethasone, colchicine, or indomethacin, inhibit neuron killing brought on by the presence of a plaque component or Aβ peptides. Activated microglia also require intracellular events signaled by signal transducers, to convert the plaque or Aβ complex formation with microglia into cellular events which lead to the production and release of neurotoxins including NTox. As shown

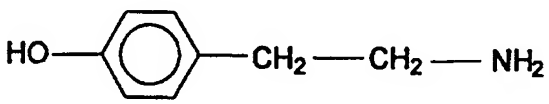
in **Figure 11**, agents that block tyrosine kinases (as opposed to other transduction pathways involving molecules such as GTP binding-proteins, protein kinase A, protein kinase C, phospholipases) prevent neuron killing. Thus, the use of the microglia-neuron assay systems allowed identification of specific drug families with value in the treatment of neurodegenerative diseases or disorders, such as Alzheimer's disease.

Plaques and Aβ peptides stimulate mononuclear phagocytes, such as microglia, to release toxic phenolic amines referred to as NTox. As shown in **Figure 12**, NTox has structural similarities with a number of other compounds which contain a phenolic group and a terminal amine. As shown in **Figure 13**, NTox released by Aβ1-42-stimulated microglia did not damage neurons in the presence of tyramine.

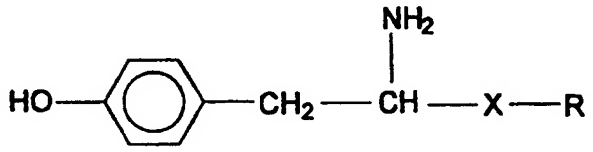
The neuroprotective effect of tyramine compounds appears selective in that other NMDA neurotoxic agonists, such as quinolinic acid, or the zwitterion AMAA are not blocked **(Figure 32)**. Since NTox has been recovered from the brains of patients with neurodegenerative diseases and disorders, including, for example, Alzheimer's disease, stroke, trauma and HIV-1 infection, tyramine compounds, as described herein, have the unique ability to prevent a selective form of neuron death, e.g., neuron death caused by NTox. See, for example, Giulian & Robertson, 1990; Giulian et al, 1993A; Giulian et al, 1995A; and Giulian et al, 1996. Generally, neurotoxins from mononuclear phagocytes are induced or activated by plaque components **(Figures 30 and 31)**.

Agents which inhibit plaque component induced or activated neurotoxicity by mononuclear phagocytes are referred to as inhibiting the effects of neurotoxins. A preferred neurotoxin inhibitor is a tyramine compound. Throughout the present disclosure and claims, the term "tyramine compound(s)" refers to and includes, for example, tyramine, tyramine derivatives, compounds of the formula (I) as described herein, compounds of the formula (II) as described herein, compounds that inhibit the toxic effects of neurotoxins, and compounds having a secondary or tertiary structure substantially similar to tyramine which are able to inhibit the toxic effects of neurotoxins

Tyramine has the following structure.



The term "tyramine compound" as used herein also includes tyrosine esters and tyrosine amides. The term "tyramine compound" also includes, for example, compounds of the formula (I):



(I)

wherein X is an ester group, an amide group, an ether group, an alkyl group having from 1 to about 20 carbon atoms or an alkyl halide group having from 1 to about 20 carbon atoms; and R is a linear, branched or cyclic, saturated or unsaturated hydrocarbon group having from 3 to about 50 carbon atoms that is optionally interrupted with one or more of an oxygen atom, a nitrogen atom, a sulfur atom or a halide atom. The levorotatory (L) form, the dextrorotatory (D) form, or the racemic mixture (DL) of a tyramine compound, including the compounds of the formula (I), may be used in the methods of the present invention.

In the compound of formula (I), X is preferably an ester group (e.g., -C(=O)O-), an amide group (e.g., -C(=O)NH-) or an ether group.

Preferably, R in the compound of formula (I) is a linear, branched or cyclic, saturated or unsaturated hydrocarbon group having from 3 to about 25 carbon atoms, more preferably from 3 to about 12 carbon atoms. Optionally, one, two, or three or more of the carbon atoms in the hydrocarbon group of R may be substituted with or interrupted by one, two or more of an oxygen atom, a nitrogen atom, a sulfur atom, and/or a halide atom (e.g., a fluorine atom, a chlorine atom, a bromine atom and/or an iodine atom). More preferably, R is a butyl group, an allyl group, a benzyl group, a naphthyl group, a long chain fatty acid, an indole group, a pyrrole group, an imidazole group, a tosyl group, a furan group, a thiophene group, a piperidine group, a phenothiazine group, a benzodiazepam group or a muscarine group. Most preferably, R is a t-butyl group, an allyl group, a benzyl group or a naphthyl group. In formula (I), R should have at least 3 carbon atoms in order to effectively block NTox activity (**Figures 30 and 31**).

Preferred compounds of formula (I) include, for example, tyrosine butyl ester, tyrosine allyl ester, tyrosine benzyl ester and tyrosine β-naphthylamide, more preferably

L-tyrosine t-butyl ester, L-tyrosine allyl ester, L-tyrosine benzyl ester and L-tyrosine β-napthylamide.

Generally, modifications which make tyramine more lipophilic result in compounds that are potent inhibitors of NTox. Structure and activity profiles show that modifications on the α-carbon of tyramine increase the neurotoxic blocking activity of the compound, while changes at the β-carbon of tyramine or the 3-OH position on the phenolic ring of tyramine reduce the neurotoxic blocking activity (**Figure 30**).

In other embodiments of the present invention, the phenol structure in the compound of formula (I) may be replaced with appropriate substituents that mimic the 4-OH benzene structure. For example, the phenol structure in formula (I) may be replaced with an indole group, a pyrrole group, an imidazole group, a tosyl group, a furan group, a thiophene group as well as other multicyclic and heterocyclic groups known in the art. Accordingly, "tyramine compounds" also includes compounds of the formula (II):

$$Y\text{-}CH_2\text{-}CH(NH_2)\text{-}X\text{-}R$$

(II)

wherein Y is an indole group, a pyrrole group, an imidazole group, a tosyl group, a furan group, or a thiophene group; X is the same as defined above in formula (I); and R is the same as defined above in formula (I). Y may be other multicyclic or heterocyclic groups known in the art.

Additionally, a tyramine compound or tyramine activity may be measured by similar hydrophobicity, hydrophilicity, acidity, and basicity of the agent or side chains thereof. Minor variations in the groups and side chains identified herein are considered to be substantially similar to tyramine. A composition comprising tyramine compounds may be used in a similar therapeutic and pharmaceutic manner as set forth above for the plaque suppressor agents.

The tyramine compounds of the present invention, including the compounds of formula (I) and (II), may be in the form of a pharmaceutically acceptable salt. "Pharmaceutically acceptable salt" refers to an inorganic acid addition salt such as hydrochloride, sulfate, and phosphate, or an organic acid addition salt such as acetate, maleate, fumarate, tartrate, and citrate. Examples of pharmaceutically acceptable metal salts are alkali metal salts such as sodium salt and potassium salt, alkaline earth metal salts such as magnesium salt and calcium salt, aluminum salt, and zinc salt. Examples of

pharmaceutically acceptable ammonium salts are ammonium salt and tetramethyl-ammonium salt. Examples of pharmaceutically acceptable organic amine addition salts are salts with morpholine and piperidine. Examples of pharmaceutically acceptable amino acid addition salts are salts with lysine, glycine, and phenylalanine. Accordingly, a pharmaceutically acceptable salt of the compound of formula (I) may be, for example, L-tyrosine allyl ester *p*-toluenesulfonate.

Tyramine compounds, including the compounds within the scope of formula (I) and formula (II), are available from a wide variety of commercial suppliers, including, for example, the Sigma Chemical Company (St. Louis, MO). Alternatively, the tyramine compounds of the present invention can be made using standard synthetic organic chemistry techniques that are well known to one skilled in the art.

The amount of the tyramine compound to be administered depends on the disease being treated, the method of administration, and the age, sex, weight and physical condition of the patient. Generally, treatment is initiated with small dosages, which can then be increased by small increments until the optimum effect under the circumstances is achieved. For *in vitro* applications, the amount of the tyramine compound to be administered may variably range from about 0.01 µg/L to about 50 µg/l, preferably from about 0.1 µg/l to about 10 µg/l, more preferably about 2.5 µg/L. For *in vivo* applications, the amount of the tyramine compound to be administered may variably range from about 0.01 µg/kg of body weight to about 100 µg/kg of body weight, preferably from about 1 µg/kg of body weight to about 50 µg/kg of body weight.

The methods of the present invention can involve either *in vitro* or *in vivo* applications. In the case of *in vitro* applications, including cell culture applications, the compounds described herein can be added to the cells in cultures and then incubated.

Additionally, the compounds of the present invention may serve as screening agents for the identification of other classes of therapeutic compounds that interfere with the toxic effects of neurotoxins. For example, tyramine compounds of the present invention may be used in binding assays, including the assays described herein, to identify other compounds that could effectively inhibit the toxic effects of neurotoxins that are released from mononuclear phagocytes. For example, the present invention may be used to identify an agent that inhibits the toxic effects of neurotoxins. First, a neuron is contacted with a neurotoxin and an agent suspected of inhibiting the toxic effects of the

neurotoxin. Thereafter, neuron function is compared to a measured control, which is obtained by contacting a neuron with a neurotoxin and a compound that inhibits the toxic effects of neurotoxins, including the tyramine compounds of the present invention. The tyramine compounds or agent suspected of inhibiting the toxic effects of the neurotoxin may be detectably labeled, as described herein. As one skilled in the art would recognize in view of the present disclosure, an agent that inhibits the toxic effects of neurotoxins is found when there is an increase in neuron function when compared to the neuron function measured in the control or when the neuron function is comparable to the neuron function measured in the control. Neuronal damage and the measure of neuron function is defined herein and may be determined or measured by, for example, determining a reduction in cell number, determining changes in biochemical markers such as loss of cell metabolic function (including changes or disruption in glucose metabolism, ATP production, ion gradient maintenance across cell membranes, protein synthesis, nucleic acid synthesis and mitochondrial respiration) release of intracellular material, penetration of impermeant dyes, reduction of the number of neurons, loss of neurofilament or synaptophysin, release of lactate dehydrogenase or other evidence of cell injury. One skilled in the art could conduct the appropriate binding assay in view of the present disclosure.

The present invention provides for other methods of identifying agents that inhibit the toxic effects of neurotoxins, such as competitive binding assays. For example, a neuron is contacted with a neurotoxin, at least one agent suspected of inhibiting the toxic effects of the neurotoxin, and a known compound that inhibits the toxic effects of neurotoxins, including the tyramine compounds of the present invention. Preferably, two or more agents suspected of inhibiting the toxic effects of the neurotoxin are used for rapid screening or identification. Further, the tyramine compounds and/or unknown agents may be detectably labeled, as described herein. Thereafter, the inhibition of the agent is compared with the inhibition of the tyramine. If there is no inhibition by the agent, then it is known that the agents do not inhibit the toxic effects of the neurotoxin. If inhibition by the agents is detected, the agents can be individually screened, as described above. In view of the present disclosure, one skilled in the art could conduct the appropriate competitive binding assay.

Additionally, one skilled in the art would recognize, in view of the present disclosure, that the tyramine compounds of the present invention may be used to identify

new classes of receptors where neurotoxins act in order to identify new biological targets for drug development.

To better serve as screening tools or identification agents, the tyramine compounds, as described herein or a compound which inhibits the toxic effects of neurotoxins, may be modified to include detection labels, including radioactive (^{125}I, ^{14}C, ^{3}H, ^{35}Sulfate), immunoconjugate (biotinylation), fluorescent (rhodamine, fluorescein), colormetric (peroxidase) and antibody detection labels. Data shows, for example, that an iodinated tyramine compound, such as, for example, tyrosine t-butyl ester, retains its neuroprotective activity (**Figure 35**).

As described in detail herein, the compounds of the present invention may be administered to a patient to inhibit the toxic effects of neurotoxins. The compounds of the present invention may be administered to a patient to treat neurodegenerative diseases or disorders including, for example, Alzheimer's disease, HIV-1 infection, AIDS dementia, amyotrophic lateral sclerosis (ALS or Lou Gehrig's disease), stroke, trauma, hereditary hemorrhage with amyloidosis-Dutch type, cerebral amyloid angiopathy, Creutzfeld-Jakob disease, Parkinson's disease and multiple sclerosis. The compounds may be administered in a pharmaceutically effective amount to inhibit the toxic effects of neurotoxins on neurons and/or in a pharmaceutically effective amount to treat neurodegenerative diseases or disorders. The compounds of the present invention may also be administered as pharmaceutically acceptable salts.

The compounds of the present invention may also be used as a neurotoxin assay in a patient, which can used to diagnose a neurodegenerative disease or disorder in the patient. In other words, the compounds of the present invention may be used as an early detection method to identify individuals who are at risk for developing neurodegenerative diseases or disorders in view of their age, family history, early symptoms or other risk factors. For example, a sample, such as blood, cerebrospinal fluid or tissue, may be taken from a patient and evaluated with the tyramine compounds of the present invention and a binding assay, as described herein, to identify the presence of neurotoxins in the patient or to identify patients who may suffer from an immune-mediated neurodegenerative disease or disorder. The patient's sample may be compared to a control to determine whether elevated levels of neurotoxins are present.

Similarly, the compounds of the present invention may be used to monitor a patient's treatment or the rate of progression of a disease by determining the amount of neurotoxins that are present in the patient's system before and throughout treatment. The compounds may also be used to monitor neurotoxin levels to allow for the adjustment of drug doses. The monitoring may be conducted using the binding assays described herein.

For example, the present invention provides methods for neurotoxin assays in a patient by contacting a patient's sample with a neurotoxin and a compound that inhibits the toxic effects of neurotoxins, such as the tyramine compounds of the present invention. Thereafter, the amount of inhibition of the tyramine compound is compared to a measured control. There is an increase of neurotoxins in the patient when there is an increase in the neurotoxin level compared to the control. The control may be based on a population of a similar age or risk factor to the patient being tested or the control may be the base-line figure for the patient upon an initial monitoring.

As will be discussed more fully below, the present invention also describes a unit dosage of tyramine compounds in a pharmaceutically acceptable carrier or diluent.

With respect to *in vivo* applications, the compounds described herein can be administered to a patient in a variety of ways including, for example, parenterally, orally or intraperitoneally. Parenteral administration includes administration by the following routes: intravenous, intramuscular, interstitial, intra-arterial, subcutaneous, intraocular, intrasynovial, transepithelial, including transdermal, pulmonary via inhalation, opthalmic, sublingual and buccal, topical, including ophthalmic, dermal, ocular, rectal, and nasal inhalation via insufflation or nebulization.

The compounds may be orally administered, for example, with an inert diluent or with an assimilable edible carrier, they may be enclosed in hard or soft shell gelatin capsules, or they may be compressed into tablets. For oral therapeutic administration, the active compounds may be incorporated with an excipient and used in the form of ingestible tablets, buccal tablets, troches, capsules, sachets, lozenges, elixirs, suspensions, syrups, wafers, and the like. The pharmaceutical composition comprising the active compounds may be in the form of a powder or granule, a solution or suspension in an aqueous liquid or non-aqueous liquid, or in an oil-in-water or water-in-oil emulsion.

The tablets, troches, pills, capsules and the like may also contain, for example, a binder, such as gum tragacanth, acacia, corn starch or gelating, excipients, such as

dicalcium phosphate, a disintegrating agent, such as corn starch, potato starch, alginic acid and the like, a lubricant, such as magnesium stearate, and a sweetening agent, such as sucrose, lactose or saccharin, or a flavoring agent. When the dosage unit form is a capsule, it may contain, in addition to materials of the above type, a liquid carrier. Various other materials may be present as coatings or to otherwise modify the physical form of the dosage unit. For instance, tablets, pills, or capsules may be coated with shellac, sugar or both. A syrup or elixir may contain the active compound, sucrose as a sweetening agent, methyl and propylparabens as preservatives, a dye and flavoring. Of course, any material used in preparing any dosage unit form should be pharmaceutically pure and substantially non-toxic. In addition, the active compound may be incorporated into sustained-release preparations and formulations.

The active compounds may be administered parenterally or intraperitoneally. Solutions of the compound as a free base or a pharmaceutically acceptable salt can be prepared in water mixed with a suitable surfactant, such as hydroxypropylcellulose. Dispersions can also be prepared in glycerol, liquid polyethylene glycols, and mixtures thereof, and in oils. Under ordinary conditions of storage and use, these preparations may contain a preservative to prevent the growth of microorganisms.

The pharmaceutical forms suitable for injectable use include sterile aqueous solutions or dispersions and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersions. In all cases, the form must be sterile and must be fluid to the extent that easy syringability exists. It may be stable under the conditions of manufacture and storage and must be preserved against the contaminating action of microorganisms, such as bacteria and fungi. The carrier can be a solvent or dispersion medium containing, for example, water, ethanol, polyol (for example, glycerol, propylene glycol, and liquid polyethylene glycol, and the like), suitable mixtures thereof, and vegetable oils. The proper fluidity can be maintained, for example, by the use of a coating, such as lecithin, by the maintenance of the required particle size (in the case of a dispersion) and by the use of surfactants. The prevention of the action of microorganisms can be brought about by various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, sorbic acid, thimerosal, and the like. In many cases, it will be preferable to include isotonic agents, for example, sugars or sodium chloride.

Sterile injectable solutions are prepared by incorporating the active compound in the required amount in the appropriate solvent with various of the other ingredients enumerated above, as required, followed by filtered sterilization. Generally, dispersions are prepared by incorporating the various sterilized active ingredients into a sterile vehicle which contains the basic dispersion medium and any of the other ingredients from those enumerated above. In the case of sterile powders for the preparation of sterile injectable solutions, the preferred methods of preparation are vacuum drying and the freeze drying technique.

Pharmaceutical compositions which are suitable for administration to the nose or buccal cavity include powder, self-propelling and spray formulations, such as aerosols, atomizers and nebulizers.

The therapeutic compounds of this invention may be administered to a mammal alone or in combination with pharmaceutically acceptable carriers or as pharmaceutically acceptable salts, the proportion of which is determined by the solubility and chemical nature of the compound, chosen route of administration and standard pharmaceutical practice.

The compositions may also contain other therapeutically active compounds which are usually applied in the treatment of the diseases and disorders discussed herein. Treatments using the present compounds and other therapeutically active compounds may be simultaneous or in intervals.

As set forth herein, the primary signal for plaque induction of microglial neurotoxicity is the Aβ peptide. Plaque fragments from AD brain induce microglia to become neurotoxic, however only those solubilized plaque fractions which contained Aβ1-42 (or Aβ1-40) stimulate both rat and human microglia to take on reactive morphologies and become neurotoxic. Testing with synthetic peptides confirmed that the Aβ1-40 and Aβ1-42 peptides were inducers of neuron-killing microglia. Other forms of Aβ, including the peptides Aβ1-28, Aβ12-28, and Aβ17-43, were inactive. The physical state of the amyloid is not related to toxicity induction since rodent Aβ1-42, which does not induce toxicity, forms the same β-pleated sheets as does human Aβ1-42 (Fraser et al., 1992). In addition, Aβ17-42, which is even more prone to aggregation than is Aβ1-42 (Pike et al., 1995; TABLE 2), is also unable to induce toxicity. In fact, testing a variety of peptide fragments shows that the N-terminal and C-terminal regions appear to play

separate and necessary roles in microglial activation. The interactions of microglia with peptide-coupled beads reveal that the N-terminus region is necessary, for anchoring of the peptide to the cells. This finding may account for the inability of rodent Aβ to induce neurotoxicity, since the first 16 amino acids of rodent Aβ are unlike the human Aβ domain. Interestingly, residues 1-16 compose the hydrophilic portion of the molecule and thus may be accessible for microglial attachment to the plaque. Without this attachment domain, Aβ is unable to induce toxicity and, in this way, prevented Aβ17-42 from activating microglia. The C-terminal portion of Aβ remains necessary to toxicity induction, however, since the N-terminus (1-16) alone was unable to induce microglial neurotoxicity.

It is important to note that the Aβ effects on microglial neurotoxicity set forth in the present invention are distinct from the *direct* neuron killing effects of Aβ described by other laboratories (Yankner, 1990; Pike et al., 1991, 1993; Cotman et al., 1992). Most laboratories exploring a direct toxicity carefully describe those specific cell culture conditions, or particular protocols for Aβ peptide preparation, which have been essential to create an environment for cell killing (Pike et al., 1991, 1993; Mattson et al., 1992; Howlett et al., 1995; Pollack et al., 1995). For example, low cell numbers appear to be necessary to demonstrate direct killing by Aβ, with cell densities typically less than 100 per mm^2 (Mattson and Rydel, 1992). In addition, toxic effects were only seen by other skilled artisans if cultures are exposed to the peptide after a defined period of incubation *in vitro* (Yankner et al., 1990), if glia are poisoned (Pike et al., 1995), if batch to batch variability among synthetic peptides is considered (May et al., 1992), if synthetic peptides are "aged" (Pike et al., 1991, 1993), or if glutamate or other glutamate receptor agonists are present (Koh et al., 1990; Mattson et al., 1992). Unfortunately, specific labeling for microglia (which may compose 5% to 10% of cells in embryonic rat hippocampal cultures) is seldom used, so the contribution of neurotoxic microglia to these other culture systems cannot be assessed.

The *in vitro* preparations useful in the present invention were optimized to maintain healthy and long-lived neuron/astroglia cultures, that were controlled to examine the role of microglial interactions with neurons. These cultures differed in several important ways from assays described by other investigators. First, the neurotoxicity assays employ high density cultures, an order of magnitude greater than the low density

systems used by others. This condition is in agreement with Mattson and Rydel (1992), where no directly toxic effects of Aβ are observed. Secondly, the culture system is very supportive of neuronal growth as shown by extensive neuritic projections and viability for several weeks beyond the test period. In culture preparations poisoned with mitotic inhibitors or in very low density cultures, neuronal survival drops by at least 50% spontaneously, as observed by Mattson and Rydel, 1992, making it very difficult to monitor and interpret the effects of any cytotoxic agent. Thirdly, microglial content of the culture system must be clearly demonstrated, since the endogenous population of microglia present in primary hippocampal cultures is fully capable of arousal to neurotoxicity by Aβl-42. Finally, a combination of neuron specific markers (MAP-2 and neurofilament) are employed to allow accurate monitoring of neurons among a mixed population of cells. In contrast, phase microscopy (which is very difficult to interpret in developing brain cell cultures), release of lactate dehydrogenase into culture media (which occurs after any cell damage), or a decline in neuron specific enolase (+) cells (which includes both neurons and glia in embryonic cultures) cannot differentiate the survival of neuronal and nonneuronal cells. While both the direct and indirect neurotoxic effects of Aβ may play roles in the neuronal pathology of AD, the striking potency of Aβ to induce neurotoxic microglia suggests that indirect, immune-mediated pathways may be substantial.

The present invention offers strategies for intervention in the pathology resulting from neurotoxic microglia in AD including (1) suppression of signaling steps as neuritic/core plaques turn quiescent microglia into reactive ones, (2) inhibition of microglial synthesis and secretion of neurotoxins, and (3) the blockade of neurotoxin attack upon neurons. In pursuit of the first of these strategies, specific domains of Aβ responsible for the various steps in the Aβ induced cascade of cellular response leading to neurotoxic microglia may be manipulated. Since the cell attachment domain in the N-terminal portion of Aβ is not itself toxic, induction of neurotoxic microglia by competition with small AD peptides may be blocked. Indeed, while anti-inflammatory drugs have been recommended as beneficial for AD (Breitner et al., 1990; McGeer et al., 1990; Schnabel, 1993; Eikelenboom et al., 1994; Lucca et al., 1994), the recommended drugs could not be properly assayed until the invention of the identification methods provided herein. In addition, microglial suppressants such as chloroquine are also indicated as a

likely candidate (Giulian et al., 1989; Giulian and Robertson, 1990), since commonly used immuno-suppressants (including glucocorticoids) do not reduce neurotoxic activities of brain mononuclear phagocytes (Giulian, 1992). Finally, the neurotoxin secreted by plaque-activated microglia can be blocked by antagonists of the NMDA receptor. NMDA receptor antagonists useful for stroke, trauma, and epilepsy might now be screened for AD and may ultimately offer benefit to the AD patient. Inhibiting the Aβ activation of microglia offers a number of therapeutic interventions, all of which may slow neuronal loss in conditions associated with brain inflammation, including diseases such as Alzheimer's disease.

EXAMPLES

The following examples are illustrative only and are not intended to limit the scope of the invention.

Example 1: A Tetrapeptide Domain Within β-Amyloid Binds to Microglia and Suppresses Induction of Neurotoxicity By Alzheimer Plaques

In order to delineate the mechanisms by which Aβ plaques elicit neuron-killing microglia, the capacity of synthetic human and rodent forms of Aβ to act as microglial activators was monitored.

Rat microglia were isolated from newborn animals using the method of Giulian and Baker, 1986, with recovery of >98% homogenous population monitored by binding the fluorescent probe acetylated low density lipoprotein labeled with 1, l'-dioctadecyl-,3,3,3',3'-tetramethyl-indo-carbocyanine [Dil-ac-LDL]). Amyloid proteins were isolated from AD neocortical gray matter laden with neuritic and core plaques with final separations involving a discontinuous sucrose gradient. Amyloid cores recovered from the 1.4/1.7 M sucrose interface as fragments (15 to 25 micron diameters) were solubilized in 80% formic acid and fractionated by Superose 12 FPLC to yield native Aβ (>99% in the Aβ1-42 form) in accordance with the methods of Lucca, U., et al., 1994. Cultured neurons prepared from rat hippocampus consisted of process-bearing neurons (10-20% of total cell population) atop a bed of astroglia (>70%) mixed with microglia (5-10%). In order to eliminate microglia, cultures were exposed saporin (a ribosome-inactivating protein) coupled to acetylated LDL (ac-LDL) for 10 pg/ml for 18 hours.

Saporin-ac-LDL selectively bound to scavenger receptors and reduced microglial numbers to <0.1% of the total population, with no effect on numbers or viability of either neurons or astroglia. After 14 days *in vitro*, cultures (with a final concentration of 0.6% serum) were exposed to test substances in the presence or absence of exogenous microglia for 72 hours. At the end of this time, the cultures were fixed in 3% paraformaldehyde at room temperature for 6 hours and immuno-stained by overnight incubation with a mixture of anti-neurofilament antibodies (SMI-311, 1:150; RT-97, 1:150; Stemberger Monoclonals, Inc.) plus anti-MAP-2 (Boehringer Mannheim, 184959; 1:200) at 4°C in the presence of 2% horse serum and 0.3% triton X-100. Data were expressed as % mean survival expressed in terms of parallel untreated control cultures after scoring at least 8 randomly selected fields for each of 3 coverslips from at least 3 independent experiments. Chemical modifications of Aβ1-42 (10 μmoles/l) were carried out at room temperature for 1 to 2 hrs with 5 mM CHD in 50 mm sodium borate buffer (pH 8.9); with 1 mm TNM in 50 mm Tris buffer (pH 8.0); with 10 mm EAM in 200 mm triethylamine HCL buffer (pH 10.0); and with 1 mm DEPC in 100 mm potassium phosphate buffer (pH 6.8). Decarbethoxylation involved the addition of 1.5 M hydroxylamine (pH 7.0) at room temperature overnight. Glutamine residues were enzymatically modified with 10 μg TNG in 100 mm Tris buffer (pH 7.4) containing 200 mm ethylamine at 37°C for 2 hr. In all cases, samples were washed using ultrafiltration with Centricon 3 and added directly to cultures.

When applied at 1 μmole/l, human Aβ1-40 or 1-42 induced toxic effects upon dense hippocampal neuron cultures (1,200 cells per mm^2) that had been supplemented with microglia (500 cells per mm^2; **Figures 14A, 14B, and 14C**). Rodent Aβ1-40, which differs from human Aβ at residues 5,10, and 13, did not activate microglia (**Figure 14C**). Since these residues appeared to be necessary for induction of neuron killing, next human Aβ1-42 was chemically or enzymatically modified to mimic the rodent form. Cyclohexanedione (CHD) modification of the residue Arg5 or tetranitromethane (TNM) modification of Tyr10 had no effect upon AP activation of microglia (**Figure 27D**). In contrast, diethylpyrocarbonate (DEPC) treatment of His6, Hisl3, and Hisl4 eliminated neuron killing. Reversal of the DEPC modifications by hydroxylamine restored AP as a stimulus and confirmed to the role of His residues. Exploring further the involvement of residues neighboring His13 and Hisl4, both transglutaminase (TNG) cross-linking of Glnl5 to ethylamine, and acetimidination of Lysl6 by ethyl acetimidate (EAM) also

blocked Aβ1-42 induction of neurotoxic microglia (**Figure 14D**). The need for the 13-16 Aβ domain was confirmed by the synthetic peptide Aβ1-42$_{Gln13Gln14}$ which was unable to elicit neurotoxic responses (**Figure 14D**). Thus, a combination of techniques indicate that residues His13, His14, Gln15, and Lys16 (the HHQK domain within Aβ) are required or induction of neurotoxic microglia. This domain by itself, however, is not able to induce neuron killing (see below).

Previous studies on the conformation and solubility of synthetic Aβ peptides suggest that residues 1-16 make up a hydrophilic region which extend along the surfaces of Aβ fibrils. This hydrophilic region, moreover, is thought to provide binding sites for various plaque associated molecules. To determine whether the HHQK domain serves as a binding site to mediate Aβ interactions with reactive microglia, Aβ1-42 was coupled to fluorescent microspheres (1 μm diameters). The Spheres $_{Aβ1-42}$ were rapidly engulfed by cultured microglia and induced neuron killing in a manner similar to that of plaques or native Aβ (**Figure 14D**). All peptides (10 μmoles/l) which contained the HHQK domain (Aβ1-16, 10-16, 1-28, or 10-20) effectively prevented microglial binding to Spheres $_{Aβ1-42}$ while peptides lacking the HHQK domain (Aβ1-5, 1-11, 17-43, and 36-42) did not (**Figure 15A**). Significantly, both rodent Aβ1-40 (containing His13-Arg) and human Aβ1-42 $_{Gln13,Gln14}$ were unable to block microglial adherence to Spheres $_{Aβ1-42}$. Together, these data point to the participation of the HHQK sequence during plaque contact with microglia.

Isolated microglia (1000 per mm^2) were placed atop 16 mm glass coverslips in 24-well culture plates. Each well then received 250,000 Spheres $_{Aβ1-42}$ or Spheres$_{mal-BSA}$ in the presence or absence of Aβ peptides, GAGs, or scavenger receptor ligands. Binding assays were carried out at 37 °C for 4 hours after which coverslips were dipped 10 times in phosphate buffer and fixed with 4% buffered paraformaldehyde. Microsphere adherence to cells was scored at 200 magnification with phase/fluorescence microscopy. Data, expressed as % inhibition of sphere binding, was calculated from total number of spheres per field noted for control cultures receiving spheres only. Values are based upon at least 3 coverslips from 2 independent experiments. Fluoresbrite carboxylate YG microspheres (1.0 micron diameters; 0.5 ml of a 2.5% suspension) were activated with 1% carbodiimide for 4 hr at room temperature. Washed spheres were resuspended in 0.2 M borate buffer (pH 8.5) in the presence of 300 μg Aβ or 400 μg BSA in 6% DMSO. After overnight

mixing at room temperature, the spheres were washed extensively and blocked by 1 M glycine (pH 8.0) for 30 minutes. Maleylation of coupled BSA was carried out at room temperature by adding 1 M maleic anhydride in acetonitrile at pH 9.0.

Spheres $_{A\beta 1-42}$ binding to microglia was markedly reduced by trypsinization of intact cells (**Figure 15B**), suggesting that peptide-cell interactions were mediated by microglial surface proteins. Potential microglial binding sites include a variety of cell surface proteins which are expressed as microglia become reactive. One example is the dramatic appearance of scavenger receptors within hours after traumatic or ischemic CNS injury. Christie et al have, moreover, found an increased expression of scavenger receptors among reactive microglia associated with plaques in AD brain. To assess the role of the scavenger receptor in Aβ-microglia interactions, BSA was coupled to microspheres and then modified the coupled protein to produce maleylated-BSA (Mal-BSA), a known scavenger receptor ligand. By monitoring microglial binding to Spheres$_{A\beta 1-42}$ or Spheres $_{mal\text{-}BSA}$ in the presence of scavenger receptor ligands, it was, possible to determine whether this receptor provided a recognition site for Aβ. When microglia were incubated with Spheres $_{A\beta 1-42}$ and heparin sulfate, chondroitin sulfate, acetylated low density lipoprotein (ac-LDL), or dextran sulfate, only heparin sulfate blocked Spheres $_{A\beta 1-42}$ binding to microglia. In contrast, scavenger receptor ligands such as ac-LDL and dextran sulfate blocked Spheres $_{mal\text{-}BSA}$ binding (**Figure 15C**). Moreover, Aβ1-16 selectively suppressed Spheres $_{A\beta 1-42}$ binding but did not affect microglial interactions with Spheres $_{mal\text{-}BSA}$. Trypsinization of cells reduced binding of either type of coupled microsphere while pre-treatment of microglia with heparinase selectively eliminated Spheres $_{A\beta 1-42}$ binding (**Figure 15B**). Chrondroitin sulfatase did not alter the microglia binding to either Spheres $_{A\beta 1-42}$ or Spheres $_{mal\text{-}BSA}$. These data suggest that heparin sulfate on the surface of microglia provides an anchoring site for human Aβ which is independent of the scavenger receptor.

To determine if the heparin-containing site was actually involved in the activation of neurotoxic microglia, next the levels of glycosaminoglycans (GAGs) in hippocampal cultures was reduced by enzymatic degradation or by blockade of synthesis. Removal of the heparin sulfate from microglial surfaces by heparinase caused a small reduction in neurotoxicity during the 72 hour neuron survival assay (**Figure 15D**), while heparinase treatment plus a biosynthetic blockade of GAGS, including heparin sulfate, by 4-methylumbelliferyl-p-D-xyloside (β-D-xyloside) totally eliminated induction of neuron

killing. In contrast, chondroitin sulfatase with or without β-D-xyloside showed only a small effect upon plaque activation of microglia. The examples provided herein provide evidence for protein-associated heparin sulfate participation in plaque activation of microglia through the HHQK domain of Aβ. It is striking that residues 12 to 17 of Aβ has been previously identified as a GAG binding site and that several laboratories have found GAGs to alter Aβ accessibility to cell metabolism. The observations reported here add another role for GAGs, implicating a membrane-associated heparin sulfate the immune activation of AD brain.

Peptides which contain the HHQK domain block Aβ1-42 adherence to microglia and, thus, might also prevent Aβ1-42 induction of neurotoxic glia. To test this possibility, various peptides (each at 10 μmoles/liter) were added together with human Aβ1-42 (1 μmole/liter) to neuron cultures containing microglia. Only peptides with residues 13 to 16, namely Aβ1-28, 1-16, 10-20, 10-16, and Aβ13-16 blocked neurotoxin production while peptides Aβ1-5, 1-11, 17-43, and 36-42 did not (**Figures 16A and 17**). Similar protective effects were found when using Spheres Aβ1-42 as the inducer signal. Dose response curves (**Figure 16B**) showed two distinct patterns with the more potent blocking peptides (Aβ1-16, 10-16, and 13-16; ED_{50} about 30 nmoles/1) restricted to the hydrophilic region of Aβ; the less potent blockers (Aβ13-20 and 1-28; ED_{50} of 250 nmoles/1) included more hydrophobic regions of Aβ thought to be less accessible in the fibrillar aggregates. In contrast to Aβ1-40 or Aβ1-42, none of the blocking peptides elicited neuron killing (**Figure 14C**). Since peptides Aβ1-16, 13-20, 10-16, and 13-16 also blocked plaque-induced toxicity in vitro (**Figure 16C**), residues 13 to 16 of Aβ are essential not only for induction of neurotoxic microglia in culture but also for plaque activation of microglia in situ. Although the N-terminal domain of Aβ alone is responsible for amyloid binding to microglia, the C-terminal portion of Aβ must be present to activate neurotoxic pathways.

Example 2: Specific Domains of β-Amyloid from Alzheimer Plaque Elicit Neuron Killing in Human Microglia

(A) Isolation of Microglia

Rat microglia were isolated from newborn animals using the method of Giulian and Baker (1986) with recovery of about 0.5 x 10^6 ameboid microglia per brain with >99%

purity. Criteria used to identify mononuclear phagocytes included the presence of scavenger receptors as shown by binding a fluorescent probe (acetylated low density lipoprotein labeled with 1,1'-dioctadecyl-1,3,3,3',3-tetramethyl-indo-carbocyanine [DiI-ac-LDL]), the presence of CR3 complement receptor (labeling with OX-42 antibody), characteristic spine bearing cell surface morphology seen by scanning electron microscopy, and the ability to engulf fluorescent polystyrene microspheres (1 micron, Covaspheres Particles). Human microglia were isolated from 50 to 100 g of normal adult cortical gray matter within a 6 hour post-mortem interval as described in accordance with the procedure set forth by Giulian et al., 1995b. Cells of a high degree of purity (>98%, about 0.5 x 10^6 cells per gram wet weight of tissue) were obtained, that were active phagocytes, and that show the presence of CD4, spine bearing surface morphology, scavenger receptors, and the class II marker HLA-DR.

(B) Isolation, Purification, and Characterization of Plaque Proteins

AD brains were obtained from patients with both clinical history and pathologic features to meet diagnostic criteria of CERAD as defined by Mirra and Heyman, 1993. Amyloid proteins were isolated from AD cerebral cortex laden with neuritic and core plaques using discontinuous sucrose gradients of 1.2 M, 1.3 M, 1.4 M, 1.7 M, and 2.0 M. Amyloid cores were recovered from the 1.4/1.7 M sucrose interface as discrete, dense particles (15 to 25 micron diameters) and found to be thioflavine S(+) and 6E10 anti-amyloid antibody(+) (from Institute of Basic Research, Staten Island, NY). These purified cores were solubilized in 80% formic acid, fractionated by Superose 12 FPLC and dialyzed (1000 dalton cutoff) against 20 mM ammonium bicarbonate containing 0.7% zwitterion betaine in accordance with the procedures of Roher et al., 1993a. The protein content of each fraction prior to dialysis was determined by amino acid analysis (Roher et al., 1993a) and morphology was examined by transition electron microscopy (TEM) in accordance with the techniques of Roher et al., 1988. The resulting five fractions obtained from pooled brain samples were highly reproducible both in content and quantity as described by Roher et al., 1988, 1993a,b. Estimates of Aβ1-40 and Aβ1-42 content in plaque fractions were determined by employing techniques described by Kuo et al. (1996). Briefly, total Aβ determinations were carried out with an ELISA method (capture monoclonal antibody 266; biotinylated reporter monoclonal antibody C6C) and measurement of Aβ1-42 by a reporter antibody 277.2 (which recognized Aβ residues 36-

42) against standard curves of synthetic peptides. Estimates of Aβ1-40 were calculated as [μg total Aβ) - (μg Aβ1-42)]. The amyloid components of the plaque fractions were also characterized by tris-tricine PAGE and by western blots. The presence of (α-l-antichymotrypsin and apolipoprotein E in fractions was confirmed by immuno-staining with TEM, utilizing a colloidal gold technique as described above in accordance with the techniques set forth in Roher et al., 1993b.

Diffuse plaque proteins were isolated from postmortem brains rich in diffuse plaques (>85% diffuse deposits as viewed by thioflavine S stained sections) from patients with no clinical history of dementia. Diffuse plaque aggregates were recovered using the same methods for obtaining neuritic/core plaque fragments, but appeared as fine, thioflavine S(+), 6E10 antibody(+) threads (1 to 5 micron diameters) from the 1.4M/1.7M sucrose gradient interface. This material was then solubilized in 80% formic acid at room temperature for 30 min., centrifuged at 250,000 x g for 30 min., and sequentially dialyzed (1,000 dalton cut off) against 40 mM ammonium bicarbonate, 6% betaine, pH 7.8; against 40 mM ammonium bicarbonate, 2% betaine, pH 7.8; and against 20 mM ammonium bicarbonate, 0.7% betaine, pH 7.8. Protein content prior to dialysis was estimated by measuring total amine content using the fluorescamine assay upon acid hydrolyzates (6 N HCL; 24 hours; 105°C). Solubilized proteins from diffuse or neuritic/core plaques were added to cultures at a concentration of 400 μmoles/l total amine. All synthetic peptides were purchased from California Peptide (Napa, CA) or Bachem (King of Prussia, PA).

(C) Aβ Coupling to Beads and Cell Adherence Assays

Synthetic Aβ peptides were linked to Sepharose beads (Pharmacia) in coupling buffer (0.1 M $NaHCO_3$, 0.5 M NaCl, pH 8.3) containing 20% DMSO. This solution was combined with an appropriate volume of CNBr-activated Sepharose 4B (10 mg protein per ml of bead; Pharmacia protocol) and mixed overnight on a platform mixer at room temperature. Remaining active groups on the beads were blocked by 1.0 M glycine and excess uncoupled peptides removed from this bead-peptide complex by washing with three cycles of alternating pH (0.1 M acetate buffer, pH 4.0, followed by 0.1 M Tris-HCL buffer, pH 8.0, each buffer containing 0.5 M NaCl). Coupled beads (10^4 per ml) were placed in 35 mm culture dishes covered with 250,000 adhering microglia at 37°C in 1.5 ml N_2 medium in accordance with the procedure of Giulian et al., 1995a. The numbers of microglia which detached from the plate and bound to coupled beads were determined at

the end of a 6 hour incubation period by inverted phase microscopy with 100 beads scored from each of three sister cultures. Glycine and bovine serum albumin (BSA) coupled beads were used as controls.

Fluoresbrite carboxylate microspheres (YG 1.0 μm, Polysciences; 0.5 ml of a 2.5% suspension) were washed twice in 0.1 M carbonate buffer (pH 9.6) and three times in 0.02 M sodium phosphate (pH 4.5). Carbodiimide was then added dropwise to a final concentration of 1% and the suspension mixed for 4 hours at room temperature. Following 3 more washes in 0.02 M sodium phosphate, the pellet was resuspended in 1.2 ml of 0.2 M borate buffer (pH 8.5) and 300 μg Aβ (or control peptide) and 5% DMSO added. After overnight mixing at room temperature, the microspheres were blocked by 1 M glycine (pH 8.0) for 30 minutes and washed in 10 mg/ml BSA in phosphate buffer.

Example 3: Neuron Cultures and Toxicity Assays - Establishing Cultures To Study Microglia-Neuron Interactions

Cultured neurons prepared from rat hippocampus were used to assay for neurotoxins in accordance with the methods of Giulian et al., 1995a. Briefly, hippocampal cells obtained from embryonic stage 18 rat fetuses were dispersed mechanically in the presence of 0.125% trypsin and plated onto poly-L-lysine coated glass coverslips in 24 well plates at 250,000 cells per well (resulting in adhering cell densities of about 1250 cells per mm^2) in chemically-defined N2 culture media containing 5% fetal bovine serum. Gradual reduction of serum began on day 10 *in vitro* with daily 1:1 volume replacements with chemically-defined media, until a final concentration of 0.6% serum was achieved. Mature cultures consisted of process-bearing neurons (15% to 20% of total cell population) atop a bed of astroglia (>75%) mixed with microglia (from 3% to 8%; see **Figure 18**). In order to eliminate microglia, cultures were exposed to saporin (sap; a ribosome-inactivating protein; Davis and Wiley, 1989) coupled to acetylated LDL (ac-LDL) at a concentration of 10 μg/ml for 12 hours. Saporin-ac-LDL selectively bound to scavenger receptors and reduced microglial numbers to <0.1% of the total population, but had no effect on numbers or viability of either neurons or astroglia (**Figure 18**). Cultures depleted of microglia were then exposed to test substances in the presence or absence of exogenous microglia (100,000 cells per culture). Typically, the microglia:neuron ratio at the time of assay was about 3:1 and the astroglia:neuron ratio about 6:1. Controls

included cultures without saporin-treatment, cultures without test substances, and cultures with test substances but lacking microglia.

On day 14 of culture, immediately following saporin treatment, test substances were added to the hippocampal cultures. Synthetic peptides were supplied as 1 mM stock solutions in 0. 1 M phosphate buffered saline, pH 7.2, stored for at least 1 week at 4°C before use. After 72 hours incubation, the cultures were fixed in 4% paraformaldehyde at room temperature for 18 hours and immuno-stained by overnight incubation with a mixture of anti-neurofilament antibodies (NF; SMI-311, 1:600; Stemberger Monoclonals, Inc.) plus anti-MAP-2 (Boehringer Mannheim, 184959; 1:600) at 4°C in the presence of 2% horse serum and 0.3% triton X-100 to delineate both neuronal cell bodies and neurites. Astroglia were visualized using sheep anti-glial fibrillary acidic protein (GFAP, Sigma) as described by Giulian et al., 1989. Finally, to label all cell nuclei, the coverslips were exposed to 0.01% bisbenzimide (Hoechst 33258; Sigma) in PBS, pH 6.9, and rinsed in distilled water and mounted in glycerin. Immuno-labeled cells per field and nuclei per field were scored at 200X magnification using fluorescence microscopy. Data were expressed as % mean survival expressed in terms of parallel untreated control cultures after scoring at least 20 randomly selected fields for each of 3 coverslips.

Neuron free cultures were prepared from postnatal day 7 rat optic nerve. After 3 days in N2 culture media supplemented with 5% fetal bovine serum, cells were maintained an additional 4 days in serum free N2 medium. Cultures were then fixed and immunostained for neuronal and astroglia markers as described for hippocampal cells. Additionally, oligodendroglia containing galactocerebroside were immunostained with the monoclonal antibody 01 in accordance with the methods of Sommer and Schachner, 1981; Bansal et al., 1989; provided by Pamela Knapp, University of Kentucky, and prepared in accordance with standard methods of preparing monoclonal antibodies, such as that of Kohler & Milstein, 1975). Immuno-staining of neuron specific enolase was carried out in a 1:2000 dilution of rabbit anti-rat polyclonal antibody (Polysciences, Inc.) overnight at 4°C, after rinsing in 50 mM glycine, 0.3% triton X-100, and 10% horse serum.

Ciliary neurons from embryonic day 9 chick embryos were plated onto poly-L-lysine coated coverslips in 24 well plates at 2 ganglia per well in N2 media (diluted to 90%) and supplemented with 30 mM KCl plus 0.6% horse serum (modified from Giulian et al., 1993b). Cultures consisted of about 50% neurofilament(+) neurons mixed with

Schwann cells and were free of mononuclear phagocytes and astroglia. Ciliary neurons were sensitive to the toxic effects of NMDA and quinolinic acid (Giulian et al., 1993a,b). Neurotoxic activity was measured after 48 hours as described in detail previously (Giulian et al., 1993a). The percent neuron kill score was calculated as [1-(neurons per field in treated group/neurons per field in the untreated control group)] x 100%. Data were expressed as mean values ± standard error, with each value obtained from 18 fields per coverslip using at least 6 coverslips per group.

Example 4: Biochemical Studies of Toxic Agents

Purification of neuron killing activity from culture media conditioned by activated microglia was performed in accordance with the methods of Giulian et al., 1995a and ultrafiltrated through a YM-30 membrane followed by a YM-1 membrane. The ultrafiltrates were then washed with equal volumes of ethyl acetate under acidic conditions (pH 4.0) and then extracted into ethyl acetate under alkaline conditions (pH 10.5). All neurotoxic activity was recovered into this basic organic phase. Material was re-extracted into an acidic aqueous phase (pH 2.0), dried under vacuum, flushed with nitrogen gas, and subjected to acid hydrolysis (in 6 N HCL for 24 hours at 105°C). Hydrolysates were then extracted into basic ethyl acetate and eluted twice from C18 RP-HPLC (3.9 x 150 mm Nova-Pak, Waters) with a 0% to 20% acetonitrile gradient developed over 35 minutes (solvent A, 0.1% trifluoroacetic acid in dH_20; solvent B, 0.1% trifluoroacetic acid in dH_20:acetonitrile 5:95, v/v). Purification of neurotoxic activity from AD neocortex involved an aqueous extract (10 vols sterile distilled water per tissue weight) from 1 kg of minced gray matter of frozen human brain which was subjected to an identical fractionation as described above, using ultrafiltration, organic extraction, acid hydrolysis, and RP-HPLC in accordance with the methods of Giulian et al., 1995a. Phenolyic and amine contents were used to estimate concentrations of neurotoxin found within highly purified HPLC fractions. Assigning a UV_{max} of 265 nm (0.1% trifluoroacetic acid in 14% acetonitrile in dH_20), peaks of activity eluted from C18-HPLC were compared to a standard curve of tyramine eluted under identical conditions measured with a multiple wavelength detector (Rainin Dynamax UV-M). Amine content was determined by the fluorescamine method using tyramine as a standard. These detection methods gave similar

values for a given toxin preparation; the estimates of toxin concentration assumed one amine and one phenolic ring per molecule as described by Giulian et al., 1995a.

Acid-catalyzed esterification of neurotoxin was carried out with 3 N HCL in n-butanol (Regis Chemical Co., Morton Grove, IL) for 60 min at 80°C, short acetylation in acetic anhydride in methanol (1:3 vol:vol; Sigma) for 1 min at 25°C and the reaction was terminated by addition of excess glycine at room temperature. Neurotoxin was also modified by excess pentafluoropropionic anhydride (PFPA; Fluka Chemie AG, Switzerland) at 60°C for 60 min, with 100 units/ml of plasma amine oxidase (amine:oxygenoxidoreductase; 1.4.3.6; Worthington Biochemical Corp., Freehold, NJ) at 25°C in 1 ml of 10 mM phosphate buffered saline (pH 7.0) for 4 hours or with 390 units of polyphenol oxidase (monophenol, dihydroxyphenylalanine: oxgenoxidoreductase; 1.14.18.1, Worthington) at 25°C in 2 ml of 10 mM phosphate buffered saline (pH 7.0) for hours. In all cases, enzymatic reactions were terminated by boiling for 15 min. Inactivated-enzyme controls were prepared by boiling prior to incubation with neurotoxins.

Nitrites and nitrates, stable byproducts of nitric oxide synthetase (NOS) served as markers for nitric oxide (NO) synthesis (Ignarro, 1990). Nitrite/nitrate concentrations in media conditioned by isolated human microglia (10^6 cells per ml; for 24 to 72 hours incubated in the presence of neuritic/core plaques or Aβ peptides) were measured by the Griess method set forth by Beckman et al., 1990 against a standard curve ranging from 0.1 to 50.0 μM nitrate.

Example 5: Establishing Cultures To Study Microglia-Neuron Interactions

Reactive microglia in AD brain cluster around neuritic and core plaques, but do not interact with diffuse ones (Perlmutter et al., 1992; Giulian et al., 1995a). Recent studies have indicted that such reactive microglia are a source of neurotoxic factors and may injure neurons in a variety of disorders (Giulian, 1992; Giulian et al., 1995a). Study of such putative glia-neuron interactions requires suitable *in vitro* methods. Current models of AD pathogenesis have relied heavily upon brain cell cultures, particularly those prepared from dissociated embryonic rat hippocampus. In order to examine microglia-neuron interactions, it was necessary to develop long term *in vitro* systems that both contained robust hippocampal neurons and allowed control of microglial populations.

Dissociated E18 hippocampal cells grew well at >1,000 cells per mm^2 in N2 media supplemented with 5% fetal bovine serum. To more closely approach a chemically defined media for study of cellular interactions, the serum supplement was reduced to a minimal level by serial dilution. Although neurons anchored themselves atop a feeder layer of astroglia within 5 days, rapid reductions in serum concentrations caused astroglia to form thin processes which, in turn, dislodged adhering neurons. To preserve the neuron-astroglia relationships, sera levels were reduced gradually beginning on day 10 in vitro by partial media changes. Under such conditions, hippocampal populations contained about, 15% neurofilament(+), MAP2(+) neurons (**Figure 18A**), about 5% scavenger receptor(+) microglia (**Figure 18C**), and >75% GFAP(+) astroglia (**Figure 18E**). The microglia present in these cultures have been previously shown to be a significant source of cytokines and cytotoxins (Giulian et al., 1989; Giulian et al., 1994; Giulian et al., 1995a). In order to eliminate microglia from these hippocampal preparations, saporin, a ribosomal inactivating protein, was coupled to ac-LDL. As shown in **Figure 18D**, saporin-ac-LDL (10 μ/ml for 12 hours) essentially destroyed all scavenger receptor(+) microglia while sparing neurons (**Figure 18B**) and astroglia (**Figure 18F**). Monitoring each of the cell populations (**Figure 18G**) confirm that this treatment brought about a selective elimination of microglia in embryonic hippocampal cultures.

Thus, saporin-ac-LDL provided a selective agent to deplete microglia; alternatively, the addition of isolated microglia (>99% homogeneity) to the neuron/astroglia cultures offered a means to selectively reestablish this glial population. By controlling mononuclear phagocyte populations, it was possible to determine if AD plaque proteins influenced microglia-neuron interactions.

Example 6: Senile Plaques and Microglial Killing of Neurons

As reported earlier, cultured microglia incubated with neuritic/core plaque fragments released neuron killing factors (Giulian et al., 1995a). To investigate the specificity of this plaque-microglia interaction, neuritic/core plaques from AD brains and diffuse plaques from normal, aged brains were isolated, solubilized, and applied to cultured hippocampal neurons in the presence or absence of microglia. As shown in **Figure 19A**, solubilized neuritic/core plaque proteins stimulated microglial release of neurotoxins, while the solubilized constituents of diffuse plaques did not. To elucidate the

signaling mechanism, solubilized neuritic/core plaque material was next fractionated into 5 major peaks by sizing chromatography (**Figure 19B**) as described previously by Roher et al. (1993a,b). Dominant constituents found in these plaque fractions]Included glycoproteins and cc-l-antichymotrypsin in peak-SI, apolipoprotein E (apoE) in S2, and significant amounts of Aβ-amyloid (predominately Aβl-42) in peaks S3, S4, and S5, as trimers, dimers, and monomers respectively (Table 1). Plaque fractions S3, S4, or S5 added to microglial cultures led to dramatic retraction of cellular processes and engulfment of amyloid (**Figures 20B and 20D**), while fractions SI and S2 had little effect upon microglial (**Figures 19C, 20A, and 20C**). The addition of plaque fractions S3, S4, and S5 to hippocampal cultures led to a severe loss of neurons, but only in the presence of microglia (**Figure 19D**). These data suggested that plaque-derived fractions S3, S4, and S5 contained f actors capable of inducing neurotoxic microglia. As shown in Table 1, Aβl-40 or Aβl-42 peptides are common to these 3 fractions and were, therefore, likely candidates as microglial activators.

Example 7: β-Amyloid Peptides and Microglial Killing of Neurons

To test whether Aβ alone could drive neurotoxic microglia, the actions of synthetic peptides were next examined (Table 2). Generally, Aβ peptides applied in PM concentrations had no damaging effects upon neurons grown in microglia-free cultures (**Figures 21A and 21C**). When, however, microglia were added to this culture system and incubated with either human Aβl-40 or Aβl-42, there was widespread neuronal loss (**Figures 21C and 22**). In the presence of 1 μmole/liter Aβ1-42, the microglial density required for maximum neuron killing was about 150 cells per mm^2 (microglia:neuron ratio of 0.8:1; **Figure 21D**), although neuron killing in the presence of <50 microglia per mm^2 was noted. Without saporin-ac-LDL pretreatment, the endogenous microglia population normally found in these cultures ranged from 40 to 80 cells per mm^2. The levels of neuron killing in preparations containing endogenous microglia (incubated with 1 μmole/liter Aβl-42) were above values predicted by cell density curves constructed from the addition of exogenous glia (**Figure 21D**), indicating that native microglia seeded with the original E18 cultures were particularly efficient as neuron killing cells. Clearly, depletion of microglia from mixed neuron-glia cultures was necessary to demonstrate-killing by inflammatory cells brought about by Aβ.

When 500 microglia per mm^2 were added to neuron cultures, Aβ1-42 and Aβ1-40 showed ED_{50}s of about 10 and 80 nmoles/l respectively (**Figure 21E**). The amount of Aβ 1-42, therefore, found in plaque fractions S3, S4, and S5 (Table 1), as well as the amounts of Aβ1-42 estimated to exist in AD brain (in the range of ng/gm tissue; Kuo et al., 1996), would be sufficient to elicit neurotoxic glia. For the most part, small Aβ peptides (Aβ1-16, Aβ1-28, Aβ1-43; Table 2) did not produce neurotoxicity in the presence or absence of microglia. An exception, however, was Aβ25-35 which was generally *cytotoxic* at high concentrations (2:30 μmoles/1) with destruction of nearly 90% of both neurons *and* glia when given at 100 μmoles/l (**Figures 21A and 21B**). This lack of neuronal specificity for high concentrations of Aβ25-35 was consistent with its damaging effects noted by others on such non-neuronal cell lines as HeLa (Pollack et al., 1995) or upon primary cultures of astrocytes (Harris et al., 1995). However, since β25-35 is not a naturally occurring biological product, it is unlikely to participate in the pathology of AD (Roher et al., 1993a,b).

A number of reports have suggested that the neurotoxic capacity of Aβ is associated with specific structural features such as aggregation or fibril formation (Pike et al., 1991, 1993). For example, Pike et al (1991) described greater neuron loss after Aβ1-42 was stored at 37°C or several days to increase the appearance of aggregates in distilled water. Both fresh and "aged" Aβ1-42 peptides prepared as described by Pike et al. (1993) were found to be equally effective in stimulating neurotoxic microglia (75.1% +- 5.8% vs. 77.5% ± 2.6% neuron kill respectively). It has been suggested that fibril formation occurring with Aβ1-42 in solution was a critical feature for its neurotoxicity (Lorenz and Yankner, 1994; Simmons et al., 1994; Howlett et al., 1995). To determine whether conformational states such as aggregation were pertinent to microglia-dependent neuron killing, various Aβ forms were covalently coupled to 1 micron fluorescent microspheres. Aβ1-42 coupled-microspheres were readily taken up by microglia in E18 cultures (**Figures 23A and 23B**), similar to the rapid cell recognition of neuritic/core plaque fragments (Giulian et al., 1995a) and native Aβ aggregates (**Figure 20**). In contrast, Aβ17-43 coupled microspheres were not engulfed by microglia (**Figures 23C and 23D**). Importantly, microsphere-coupled Aβ1-42 and Aβ1-40, as well as unbound forms in solution, activated neurotoxic microglia, while Aβ1-16 or Aβ17-43 were not effective when tested either in solution or linked to microspheres (**Figure 23E**). These observations

suggested that the primary structure of the Aβ1-42 peptide, and not such complex features as aggregation or β-sheet formation, was sufficient to induce neurotoxic glia.

Example 8: Potency of Aβ as a Direct Neurotoxin

As noted above, Aβ1-42 did not directly kill neurons when applied at 100 μmoles/liter in cultures free of microglia (**Figure 24B**) while 100 μmoles/liter AD in the presence of microglia brought about significant neuron loss (**Figure 24C**). Because previous reports (Pike et al. 1991, 1993; Cotman et al., 1992) described direct effects of Aβ1-42 upon neurons in vitro, culture conditions, other than the presence of microglia, were sought which might lead to neuron killing. Since astroglia might participate in AD mechanisms of neuronal injury, whether astroglia-free cultures of ciliary ganglia (approximately 50% neurons and 50% Schwann cells) were examined for sensitivity to Aβ. Direct application of Aβ1-42 again had no apparent effect upon survival of ciliary cells, while Aβ 1-42 stimulated microglia secreted toxin to destroy ciliary neurons (**Figure 24H**). Similarly, plaque-stimulated microglia have been found to destroy ciliary neurons (Giulian et al., 1995a).

As an alternative strategy to assess the role of astroglia in Aβ interactions with microglia, glia were eliminated from dissociated E18 hippocampal cells using mitotic inhibitors as described by Koh et al. (1990) and Pike et al. (1993). Such culture systems, however, did not provide a reliable assay for monitoring neuron killing. for these cell preparations were inherently unstable with neuron survival dropping to <80% within 48 hours. Moreover, the use of chemically-defined media or mitotic inhibitors did not actually eliminate non-neuronal cells [such as glial precursors, scavenger receptor(+) microglia, or GFAP(+) astroglia] but simply slowed glial differentiation and the degree of antigen expression. In addition, astrocytes grown under such marginal culture conditions take on a reactive morphology with long, thin processes similar to neurons. Despite reports by others (Whitson et al., 1989; Koh et al., 1990; Pike et al., 1991, 1993; Cotman et al., 1992), a reliable neuron count in such preparations was not obtained by phase microscopy. Introduction of fetal calf sera to poisoned cultures stimulated cells with neuron-like morphology to develop into GFAP(+) astroglia. Although NF(+) MAP2(+) neurons made up less than 20% of the hippocampal cell population, >90% of the cells were found to be neuron specific enolase(+). However, neuron-free cultures of developing

optic nerve also contained >85% neuron specific enolase(+) cells, including process-bearing galactocerebroside(+) oligodendroglia (**Figures 24D and 24E**) and GFAP(+) astroglia (**Figures 24F and 24G**). Overall, healthy cultures of highly enriched hippocampal neurons could not be established using methods known to skilled artisans, nor was AD toxicity within such preparations reliably interpreted. The direct action of Aβ upon primary cultures of glia-free brain neurons was not assessed.

Example 9: Specific Plaque Proteins Activate Human Microglia

Responses of human microglia to AD, as well as to other stimulants, might differ from the responses of rodent microglia. Activated rodent macrophages, for example, are richly supplied with inducible nitric oxide synthetase (iNOS) and produce cytotoxic levels of nitric oxide (NO; Lees, 1993). In the hippocampal culture system, lipopolysacchraride (2:100 μg/ml) induces rat microglial iNOS which resulted in NO-dependent killing of neurons. In this culture system, there was a dose dependent relation of nitrate/nitrite levels of the media and neuronal loss, with loss apparent at 15 μM nitrate/nitrite and above (data not shown). Human macrophages, on the other hand, are thought to contain little iNOS and produce negligible amounts of NO (Denis, 1994). For this reason, iNOS involvement in AD pathology, as recently proposed by Meda et al. (1995), remains uncertain. In order to compare responses of human cells to those of rat, human microglia was isolated from normal adult brains recovered rapidly at autopsy, (Giulian et al., 1995a,b). These human brain mononuclear cells behaved as did the rat microglia, engulfing neuritic/core plaques and retracting processes (Giulian et al., 1995a). Both the synthetic Aβ peptides and native plaque fractions S3, S4, and S5 induced human microglia to become neurotoxic (**Figures 25A and 25B**) in patterns identical to those of rat microglia. Although Aβ1-40 and Aβ1-42 were very potent inducers of neurotoxic human microglia, these peptides did not bring about release of nitrate or nitrites (**Figures 25C and 25D**). Neither Aβ exposure to rat microglia nor LPS exposure to human microglia elicited nitrate or nitrite levels above 1.5 μM. Such observations argue against involvement of microglial iNOS in the neuronal pathology of AD. Overall, human and rat microglia responded identically to Aβ, both exhibiting neurotoxicity when in culture with intact human Aβ1-42 or Aβ1-40 peptide (**Figure 25E**).

Example 10: Aβ as an Indirect Neurotoxin

A number of cytotoxic factors have been reported to participate in Aβ neurotoxicity, including radicals (Behl et al., 1994), nitric oxide (Meda et al., 1995), cytokines (Mrak et al., 1995), and NMDA-like molecules (Giulian et al., 1995a). To determine if short-lived factors play a role in Aβ induced neuron killing microglia, neuronal loss was compared when microglia were either mixed among neurons (contact) or separated from neurons by placement in filter bottomed Millex-cell chambers (no contact). Aβ1-40 and 1-42, as well as the native plaque fractions, stimulated microglia to destroy neurons despite segregation of microglia and neurons. These observations rule out involvement of short-lived free radical intermediates, since such agents required close proximity between secretory and target cells. Moreover, there was no reduction of microglia-mediated neuron killing after exposure of either human or rodent cells to Aβ upon incubation with such free radical scavengers as vitamin E, catalase, or glutathione or with such potent inhibitors of iNOS as diphenyl iodonium (DPI) or L-N-5-(1-imino-ethyl)-ornithine hydrochloride (L-NIO; **Figure 26A**). Although glutamate antagonists acting upon non-NMDA receptor sites did not protect neurons, NMDA receptor antagonists (**Figure 26B**) including AP5, AP7, MK-801, and ifenprodil prevented neuronal loss when applied at low concentrations (10 μM).

The neurotoxin recovered from Aβ1-40 or Aβ1-42 stimulated human microglia withstood boiling, showed a low molecular mass (<1000 daltons), extracted into ethyl acetate at pH 10.5, and bound to cationic exchangers such as SP-Sephadex C25, as described for plaque exposed microglial toxin in previous work (Giulian et al., 1995a). Each of these properties is shared by the neurotoxic phenolic amine which can be extracted from AD brain. Inactivation of both the microglia-derived and brain-derived neurotoxin by PFPA, fluorescamine, and plasma amine oxidase suggested the presence of a terminal amine group at the active site while insensitivity to acidified butanol esterification indicated a lack of carboxy groups (Giulian et al., 1995a). Overall, the active principal derived from Aβ-stimulated microglia exhibited properties identical to those of the neurotoxin recovered from AD gray matter or from culture media of plaque-stimulated microglia (Giulian et al., 1995a). Protease insensitivity and resistance to acid hydrolysis (6 N HCL, 105 °C, 24 hrs) of the neurotoxin ruled out peptide factors, including cytokines and Aβ. Co-elution on tandem ion exchange columns confirmed the identical

character of microglia-derived and AD brain-derived lipophilic killing factor. As shown in **Figure 26C**, a single peak of biologic activity from Aβ-stimulated microglia co-eluted with the toxin extracted from AD brain by RP-HPLC. Previous study has shown that this purified agent was an effective toxin against hippocampal neurons in vitro or in vivo in the picomolar range (Giulian et al., 1995a).

Example 11: Specific Aβ Domains Bind To Microglia

How Aβ peptides might activate microglia is addressed herein. Because adherence to plaques might serve as an important first step in the recruitment of reactive glia, it was reasonable to consider Aβ as a potential anchoring site for microglia upon plaque surfaces. To test this hypothesis, synthetic Aβ peptides or native plaque-derived proteins were covalently coupled to 90 micron Sepharose beads. These beads were then floated atop cultured dishes. Within 30 min, microglia began to detach from the culture dish and anchored to beads which were covalently coupled to native plaque proteins or synthetic Aβ1-42 (**Figures 27A and 27B**). Within 6 hours, the number of microglia adhering to plaque-protein-coated beads had increased by 5-fold when compared to cells adhering to control beads coupled to glycine or BSA (**Figure 27C**). Interestingly, Aβ peptides which contained N-terminal residues, such as Aβ1-28, also promoted cell binding, while the C-terminal portion (Aβ17-43) did not (**Figure 27C**).

To delineate further which portions of the Aβ peptide served as a microglial binding site, a variety of synthetic peptides coupled to 1 micron diameter microspheres was next compared. Within 4 hours of incubation, marked glial binding to microspheres coupled to Aβ1-42, Aβ1-40, Aβ1-16, or Aβ12-28 occurred (**Figures 28A through 28F**), with little cell binding of spheres coupled to Aβ17-43, Aβ25-35, Aβ36-42, or to rodent Aβ1-40 ($5_{Arg-Gly}$, $10_{Tyr-Phe}$, $13_{His-Arg}$) was observed. Since structural differences between the human and rodent forms of Aβ occur between residues 5 and 13 of the N-terminus, properties of this specific domain were focused upon. As shown in **Figure 28G**, Aβ12-28-microspheres provided an anchoring substrate for cells whereas Aβ1-11 did not. Examination of a heptapeptide confirmed a microglial binding domain between residues I 0 to 16 (**Figure 28G**).

The importance of the N-terminal cell binding domain for Aβ-microglial interactions was supported by the fact that neither Aβ17-43 nor $Aβ1-40_{rodent}$ induced

neurotoxic cells despite test concentrations 100-fold above that required for human AP 1-40 (**Figures 21E and 25E**). The patterns of binding and toxicity predicted that the 10 to 16 binding region would be necessary component for activation of neuron-killing microglia. To test this hypothesis, AβlO-42 was next incubated at increasing concentrations and found it to be nearly as potent as Aβl-42 in eliciting microglia-dependent killing of cells (**Figures 21E and 29A**). However, the AβlO-16 binding domain or the 17-43 region by themselves did not injure neurons (**Figure 29A**). Thus, the N terminus of human Aβ (particularly residues 10-16) was necessary, though not sufficient, for eliciting neurotoxic microglia (**Figure 29B**).

Example 12: Neurotoxin Assay and Drug Testing Methods

The procedures described above and by Giulian et al, 1996, provided for the identification of tyramine compounds as therapeutic agents for neurodegenerative diseases or disorders including, for example, Alzheimer's disease. Cells from the brain of fetal rats were grown in tissue cultures. Microglial populations within the cultures were controlled following the method described in Example 5 above. Known amounts of Aβ peptides coupled to microspheres were added to the culture. The Aβ spheres stimulated the microglia to release NTox, which in turn destroyed the neurons. The neurons in the cultures were monitored for survival by immuno-staining.

Generally, drug assays involve the addition of known concentrations of a test agent over a range of concentrations. After 72 hours, the experiment is stopped and the neurons are identified by immuno-staining. The data is expressed as % neuronal survival {1-(neuronal number in test sample/neuronal number in untreated control sample)} x 100%. Dose response curves are then used to estimate Effective $Dose_{50\%}$ (ED_{50}) of neuroprotective agents. Drug targets with an ED_{50} of less than 10 μM are generally considered good candidates for further development.

As discussed above, microglia release a toxin, referred to as NTox, when stimulated with senile plaques or Aβ peptides. As shown in Figure 1, NTox has structural features similar to tyramine (**Figures 12 and 33**). Tyramine is a non-toxic, natural product found in the brain. Such similarities suggest that tyramine or similar molecules might mimic NTox by interacting with neurons. Since tyramine itself is non-toxic, it would compete with NTox, and in this way, prevent the action of the neurotoxic factor.

As shown in **Figure 30**, a number of compounds were tested for their ability to protect neurons from damage by neurotoxic microglia which release NTox. Microglia were stimulated with Aβ1-42 and the killing of neurons was monitored after 72 hours. In this experiment, about 65% of the neurons were destroyed by the Aβ-stimulated microglia. Incubations with 10 μM tyrosine had no protective effect, while 10 μM of tyramine completely prevented neuron death. Other tyramine compounds, including tyrosine esters, were neuroprotective (**Figure 30**).

Dose response curves (**Figure 31**) showed that some tyramine compounds had greater potency than tyramine itself. For example, the ED_{50} of tyramine was about 200 nM compared to 2 nM of tyrosine t-butyl ester (see **Figure 33** for structures). In contrast, a shorter ester, such as tyrosine methyl ester, had no neuroprotective effect.

Example 13: Mechanisms of Neuroprotective Action

Initial studies to characterize the neuroprotective effect of tyramine compounds employed microglia-neuron co-culture assays in the presence of Aβ or isolated senile plaques. Inhibition of neuron killing in such a system could occur at several levels -- inhibition of microglial activation by Aβ or plaques; inhibition of NTox production and release by microglia; or inhibition of action of NTox upon neurons. The tyramine compounds act by the third mechanism as shown by the fact that killing action of NTox is inhibited in the presence of tyramine compounds including, for example, tyrosine t-butyl ester or similar tyramine compounds (**Figure 32**). Effective doses in the low nmolar range show the tyramine compounds to be very potent agents compared with low μmolar range of other neuroprotective drugs under development for neurological disorders. The high potency of tyramine compounds also suggest that mechanisms of action occur within a biological cascade involving signal amplification (i.e., receptor binding, enzyme inactivation).

The precise mechanism of NTox that leads to the destruction of neurons is only partially characterized. Based upon extensive testing with receptor antagonists, it has been discovered that two classes of neurotransmitter receptors are involved -- the n-methyl D-aspartate (NMDA) class of glutamine receptors and the Muscarinic (M receptors) class of cholinergic receptors. As shown in **Figure 32**, NTox can be blocked by potent NMDA antagonists such as AP5 or MK-801. The neuroprotective effects for these agents to the

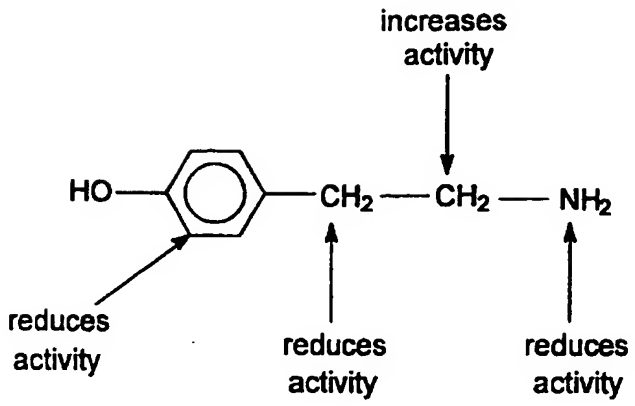
NMDA receptor are in the range of 100 nM, thus making tyramine compounds more potent and very feasible for drug development. Further study to determine if tyramine compounds act upon the NMDA receptor itself involved expression of the NMDA receptor complex in Xenopus oocytes and measurement of ionic fluxes across the oocyte membrane that are elicited by NMDA. As shown in **Figure 34**, NMDA stimulates a voltage change across the oocyte membrane that is blocked by 300 μM tyrosine t-butyl ester. Octopamine and other agents which lacked neuroprotective action in the neuron killing assays showed no effect in the oocyte assays. In addition, as shown in **Figure 32**, known NMDA receptor mediated toxins, such as quinolinic acid and AMAA, are not blocked by tyramine compounds. Thus, tyramine compounds appear to have several actions, one of which is upon the NMDA receptor.

Example 14: Structure/Function Relationship

Following the methods described herein, numerous compounds were examined to determine their neuroprotective activity. It has been experimentally found that modifying tyramine at the -OH in position 4 of the benzene ring, at the β-carbon or at the amine group, results in a compound that exhibits little or no neuroprotective activity.

Compounds which exhibit little or no neuroprotective activity include, for example, phenethylamine, dopamine, 3-OH tyramine, 6-OH dopamine, 6-OH DOPA, DOPA, methyl tyramine, octopamine, ephedrine, phenylenediamine, 4-butoxyphenol, resorcinol, tyrosine, tyrosinamide, tyrosine hydrazide, tyrosine methyl ester, tyrosine ethyl ester, acetylated tyramine, epinephrine and norepinephrine.

On the other hand, modification of tyramine with side chains and groups at the α-carbon results in compounds that enhance neuroprotective effects, enhance brain penetration and increase the stability of the compound. Such compounds include, for example, tyramine, mono- and di-iodinated tyrosine t-butyl ester, tyrosine t-butyl ester, tyrosine benzyl ester, tyrosine allyl ester (e.g., tyrosine allyl ester *p*-toluene sulfonate) and tyrosine naphthylamide (e.g., tyrosine β-naphthylamide).



Example 15: Iodinated Tyramine Compounds as Neuroprotectants

As described herein, Aβ1-42 stimulated microglia stimulate the release of NTox which destroys neurons. Tyrosine t-butyl ester was mono-iodinated and di-iodinated by a standard chloroamine T procedure, which is well-known to one skilled in the art. This procedure results in the addition of a halide (e.g., iodine) to the benzene ring. Following the method described in Example 12, it was found that mono-iodinated tyrosine t-butyl ester and di-iodinated tyrosine t-butyl ester have neuroprotective effects (**Figure 35**). Radiolabeling of tyramine compounds (e.g., ^{125}I) provides isotopically tagged reagents that can be used in binding assays to screen for new classes of neuroprotective agents and to monitor neurotoxins produced in patients, as described herein.

The disclosure of each patent, patent application and publication cited or described in this document is hereby incorporated herein by reference, in its entirety.

Various modifications of the invention in addition to those shown and described herein will be apparent to one skilled in the art from the foregoing description. Such modifications are also intended to fall within the scope of the appended claims.

Table 1: Characterization of Protein Fractions Derived From Neuritic/Core Plaques

Fraction	Major constituents	Aβ Concentrations Added to Culture (nmoles/liter; Aβ1-40, Aβ1-42)
S1	ACT[1], apoE, glycoproteins	0.1, 0.1
S2	apoE, glycoproteins	0.1, 3.2
S3	Aβ trimers[1], glycoproteins	2.0, 54.5
S4	Aβ dimers[1]	1.4, 220 *
S5	Aβ monomers1	1.0, 250 *

Major components are estimated to be ≥ 30% of total protein. Final Aβ concentrations used in neuron culture assays were based upon ELISA measurements as described in METHODS. Due to the aggregation of material in the S4 and S5 peaks, Aβ concentrations* were estimated using both amino acid analysis and ELISA for the Aβ1-40 and Aβ1-42 peptides. ACT = α-1-antichymotrypsin; apoE = apolipoprotein E; [1]Roher et al., 1993b.

Table 2: Properties of Aβ Peptides

	Principal Location	Solubility	Elicits Neurotoxic Microglia
Aβ1-28	synthetic	soluble	--
Aβ12-28	synthetic	soluble	--
Aβ17-42	diffuse deposits	very insoluble	--
Aβ25-35	synthetic	soluble	--
Aβ1-40	normal brain normal CSF vascular deposits neuritic/core plaques	moderately soluble	+
Aβ1-42	neuritic/core plaques	very insoluble	+

Properties and sources of amyloid peptides. Three major Aβ forms are known to occur in brain tissue, Aβ17-42, 1-40, and Aβ1-42. Solubility of peptides is described for chemically-defined culture media at 37°C. Aβ1-42 is the most potent stimulus for neurotoxic microglia (**Figure 17E**) and represents the major insoluble component of neuritic and core plaques.

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CLAIMS

What is claimed is:

1. A method of identifying an agent that inhibits mononuclear phagocyte-plaque component complex formation comprising:

contacting a mononuclear phagocyte with a plaque component to stimulate mononuclear phagocyte-plaque component complex formation together with an agent suspected of inhibiting said complex formation,

measuring mononuclear phagocyte-plaque component complex formation; and

comparing said measured mononuclear phagocyte-plaque component complex formation to that of a measured control,

wherein reduction of mononuclear phagocyte-plaque component complex formation compared to that of said control results in the identification of said inhibitory agent.

2. The method of claim 1 wherein said agent is a plaque suppressor.

3. The method of claim 1 wherein said plaque component is selected from the group consisting of β amyloid and active fragments thereof, α-antichymotrypsin, apolipoprotein A, apolipoprotein E, glycoproteins, heparan sulfate, and proteases.

4. The method of claim 1 wherein said plaque component is β amyloid and said agent is a plaque suppressor.

5. The method of claim 1 wherein said plaque component or said mononuclear phagocyte is adhered to a solid support to form an plaque component adhered solid support.

6. The method of claim 5 wherein said solid support is selected from a microsphere, liposome, sepharose, and sephadex.

7. The method of claim 5 wherein said plaque component is β amyloid and said solid support is a microsphere.

8. The method of claim 5 wherein said β amyloid is adhered to a solid support and is infused into a mammalian brain selected from the group consisting of primate, rodent, guinea pig, dog, cat, rabbit, and pig.

9. The method of claim 1 wherein said plaque component or said mononuclear phagocyte is labeled.

10. The method of claim 9 wherein said label is selected from the group consisting of ^{32}P, ^{125}I, ^{14}C, ^{3}H, ^{35}S, biotin, fluorescein, rhodamine, peroxidase, and antibody labeling.

11. The method of claim 1 wherein said step of measuring is selected from the group consisting of imaging said mononuclear phagocyte-plaque component complex, and detecting amplified nucleic acids from said mononuclear phagocyte of said mononuclear phagocyte-plaque component complex.

12. The method of claim 1 wherein said step of measuring comprises imaging said mononuclear phagocyte-plaque component complex and observing a signal resulting from said imaging.

13. The method of claim 1 wherein said step of measuring comprises detecting amplified nucleic acids from said mononuclear phagocyte-plaque component complex.

14. The method of claim 1 wherein said plaque component is isolated from a patient suspected of having a disease selected from the group consisting of Alzheimer Disease, hereditary hemorrhage with amyloidosis-Dutch type, cerebral amyloid angiopathy, cerebral amyloid angiopathy, Down's syndrome, spongiform encephalopathy,

Creutzfeld-Jakob disease, HIV, Parkinson's disease, multiple sclerosis, amyotrophic lateral sclerosis, stroke, and trauma.

15. The method of claim 1 wherein said plaque component or said mononuclear phagocyte is adhered to a solid support to form an plaque component adhered solid support which is infused into a mammalian brain selected from the group consisting of primate, rodent, guinea pig, dog, cat, rabbit, and pig.

16. A method of identifying an agent that inhibits mononuclear phagocyte-plaque component complex formation comprising:

contacting a mononuclear phagocyte with a plaque component coupled to a solid support to stimulate mononuclear phagocyte-plaque component complex formation together with an agent suspected of inhibiting said complex formation;

measuring mononuclear phagocyte-plaque component complex formation; and

comparing said measured mononuclear phagocyte-plaque component complex formation to that of a measured control,

wherein reduction of mononuclear phagocyte-plaque component complex formation compared to that of said control results in the identification of said inhibitory agent.

17. A method of identifying an agent that inhibits mononuclear phagocyte-plaque component complex formation comprising:

contacting a mononuclear phagocyte in a mammalian brain with a plaque component coupled to a solid support to stimulate mononuclear phagocyte-plaque component complex formation together with an agent suspected of inhibiting said complex formation;

measuring mononuclear phagocyte-plaque component complex formation; and

comparing said measured mononuclear phagocyte-plaque component complex formation to that of a measured control,

wherein reduction of mononuclear phagocyte-plaque component complex formation compared to that of said control results in the identification of said inhibitory agent.

18. The method of claim 17 wherein said step of measuring is selected from the group consisting of imaging said mononuclear phagocyte-plaque component complex, and detecting nucleic acids from said mononuclear phagocyte of said mononuclear phagocyte-plaque component complex.

19. The method of claim 17 wherein said plaque component or said mononuclear phagocyte is labeled.

20. The method of claim 19 wherein said label is selected from the group consisting of ^{32}P, ^{125}I, ^{14}C, ^{3}H, ^{35}S, biotin, fluorescein, rhodamine, peroxidase, and antibody labeling.

21. The method of claim 17 wherein said step of measuring comprises imaging said mononuclear phagocyte-plaque component complex and observing a signal resulting from said imaging.

22. The method of claim 17 wherein said step of measuring comprises detecting amplified nucleic acids from said mononuclear phagocyte-plaque component complex.

23. The method of claim 1 wherein said mononuclear phagocyte is selected from the group consisting of a microglial cell, a monocyte, a macrophage, and a microglial precursor cell, monocyte precursor cell, and a macrophage precursor cell.

24. A method of identifying an agent that inhibits plaque component activation of a mononuclear phagocyte comprising:

contacting a mononuclear phagocyte with a plaque component to stimulate plaque component activation of said mononuclear phagocyte together with an agent suspected of inhibiting said activation;

measuring plaque component activation of said mononuclear phagocyte; and

comparing said plaque component activation of said measured mononuclear phagocyte to that of a measured control,

wherein reduction of plaque component activation of said mononuclear phagocyte compared to that of said control results in the identification of said inhibitory agent.

25. The method of claim 24 wherein said agent is a mononuclear phagocyte inactivator.

26. The method of claim 24 wherein said plaque component is selected from the group consisting of β amyloid and active fragments thereof, α-antichymotrypsin, apolipoprotein A, apolipoprotein E, glycoproteins, heparan sulfate, and proteases.

27. The method of claim 24 wherein said plaque component is β amyloid and said agent is a mononuclear phagocyte inactivator.

28. The method of claim 24 wherein said plaque component or said mononuclear phagocyte is adhered to a solid support to form an plaque component adhered solid support.

29. The method of claim 28 wherein said solid support is selected from a microsphere, liposome, sepharose, and sephadex.

30. The method of claim 28 wherein said plaque component is β amyloid and said solid support is a microsphere.

31. The method of claim 27 wherein said β amyloid is adhered to a solid support and is infused into a mammalian brain selected from the group consisting of primate, rodent, guinea pig, dog, cat, rabbit, and pig.

32. The method of claim 24 wherein said plaque component or said mononuclear phagocyte is labeled.

33. The method of claim 32 wherein said label is selected from the group consisting of ^{32}P, ^{125}I, ^{14}C, ^{3}H, ^{35}S, biotin, fluorescein, rhodamine, peroxidase, and antibody labeling.

34. The method of claim 24 wherein said step of measuring is selected from the group consisting of imaging said mononuclear phagocyte, detecting amplified nucleic acids from said mononuclear phagocyte, observing altered mononuclear phagocyte morphology, observing the expression of cell surface molecules on said mononuclear phagocyte, and observing the release of nitric oxide, free radicals, cytokines, lipoproteins, enzymes, and proteins from said mononuclear phagocyte.

35. The method of claim 24 wherein said step of measuring comprises imaging said mononuclear phagocyte and observing a signal resulting from said imaging.

36. The method of claim 24 wherein said step of measuring comprises detecting amplified nucleic acids from said mononuclear phagocyte.

37. The method of claim 24 wherein said mononuclear phagocyte is a mononuclear phagocyte selected from a patient suspected of having a disease selected from Alzheimer Disease, hereditary hemorrhage with amyloidosis-Dutch type, cerebral amyloid angiopathy, cerebral amyloid angiopathy, Down's syndrome, spongiform encephalopathy, Creutzfeld-Jakob disease, HIV, Parkinson's disease, multiple sclerosis, amyotrophic lateral sclerosis, stroke, and trauma.

38. The method of claim 24 wherein said plaque component or said mononuclear phagocyte is adhered to a solid support to form an plaque component adhered solid support which is infused into a mammalian brain selected from the group consisting of primate, rodent, guinea pig, dog, cat, rabbit, and pig.

39. A method of identifying an agent that inhibits plaque activation of a mononuclear phagocyte comprising:

contacting a mononuclear phagocyte with a plaque component coupled to a solid support to stimulate plaque component activation of said mononuclear phagocyte together with an agent suspected of inhibiting said activation;

measuring plaque component activation of said mononuclear phagocyte; and

comparing said plaque component activation of said measured mononuclear phagocyte to that of a measured control,

wherein reduction of plaque component activation of said mononuclear phagocyte compared to that of said control results in the identification of said inhibitory agent.

40. A method of identifying an agent that inhibits plaque activation of a mononuclear phagocyte comprising:

contacting a mononuclear phagocyte in a mammalian brain with a plaque component coupled to a solid support to stimulate plaque component activation of said mononuclear phagocyte together with an agent suspected of inhibiting said activation;

measuring plaque component activation of said mononuclear phagocyte; and

comparing said plaque component activation of said measured mononuclear phagocyte to that of a measured control,

wherein reduction of plaque component activation of said mononuclear phagocyte compared to that of said control results in the identification of said inhibitory agent.

41. The method of claim 40 wherein said step of measuring is selected from the group consisting of imaging said mononuclear phagocyte, detecting amplified nucleic acids from said mononuclear phagocyte, observing altered mononuclear phagocyte

morphology, observing the expression of cell surface molecules on said mononuclear phagocyte, and observing the release of nitric oxide, free radicals, cytokines, lipoproteins, enzymes, and proteins from said mononuclear phagocyte.

42. The method of claim 40 wherein said plaque component or said mononuclear phagocyte is labeled.

43. The method of claim 42 wherein said label is selected from the group consisting of ^{32}P, ^{125}I, ^{14}C, ^{3}H, ^{35}S, biotin, fluorescein, rhodamine, peroxidase, and antibody labeling.

44. The method of claim 40 wherein said step of measuring comprises imaging said mononuclear phagocyte and observing a signal resulting from said imaging.

45. The method of claim 40 wherein said step of measuring comprises detecting amplified nucleic acids from said mononuclear phagocyte.

46. The method of claim 24 wherein said mononuclear phagocyte is selected from the group consisting of a microglial cell, a monocyte, a macrophage, and a microglial precursor cell, monocyte precursor cell, and a macrophage precursor cell.

47. A method for identifying an agent that inhibits plaque component induced neurotoxicity of a mononuclear phagocyte comprising:

contacting a mononuclear phagocyte with a plaque component to stimulate plaque component induced neurotoxicity together with an agent suspected of inhibiting said plaque component induced neurotoxicity of a mononuclear phagocyte;

measuring plaque component induced neurotoxicity of said mononuclear phagocyte; and

comparing said measured plaque component induced neurotoxicity to that of a measured control,

wherein reduction of plaque component induced neurotoxicity compared to that of said control results in the identification of said inhibitory agent.

48. The method of claim 47 wherein said agent is an inactivator of neurotoxic mononuclear phagocytes.

49. The method of claim 47 wherein said plaque component is selected from the group consisting of β amyloid and active fragments thereof, α-antichymotrypsin, apolipoprotein A, apolipoprotein E, glycoproteins, heparan sulfate, and proteases.

50. The method of claim 47 wherein said plaque component is β amyloid and said agent is an inactivator of a neurotoxic mononuclear phagocyte.

51. The method of claim 47 wherein said plaque component or said mononuclear phagocyte is adhered to a solid support to form an plaque component adhered solid support.

52. The method of claim 51 wherein said solid support is selected from a microsphere, liposome, sepharose, and sephadex.

53. The method of claim 51 wherein said plaque component is β amyloid and said solid support is a microsphere.

54. The method of claim 51 wherein said β amyloid is adhered to a solid support and is infused into a mammalian brain selected from the group consisting of primate, rodent, guinea pig, dog, cat, rabbit, and pig.

55. The method of claim 47 wherein said plaque component or said mononuclear phagocyte is labeled.

56. The method of claim 55 wherein said label is selected from the group consisting of ^{32}P, ^{125}I, ^{14}C, ^{3}H, ^{35}S, biotin, fluorescein, rhodamine, peroxidase, and antibody labeling.

57. The method of claim 47 wherein said step of measuring is selected from the group consisting of observing a loss of metabolic function, release of intracellular material, penetration of impermeant dyes, and reduction of cell number of neurons.

58. The method of claim 47 wherein said step of measuring comprises imaging said plaque component induced mononuclear phagocyte neurotoxicity and observing a signal resulting from said imaging.

59. The method of claim 47 wherein said step of measuring comprises detecting amplified nucleic acids from said plaque component induced mononuclear phagocyte neurotoxicity.

60. The method of claim 47 wherein said plaque component is selected from a patient suspected of having a disease selected from the group consisting of Alzheimer Disease, hereditary hemorrhage with amyloidosis-Dutch type, cerebral amyloid angiopathy, cerebral amyloid angiopathy, Down's syndrome, spongiform encephalopathy, Creutzfeld-Jakob disease, HIV, Parkinson's disease, multiple sclerosis, amyotrophic lateral sclerosis, stroke, and trauma.

61. The method of claim 47 wherein said plaque component or said mononuclear phagocyte is adhered to a solid support to form an plaque component adhered solid support which is infused into a mammalian brain selected from the group consisting of primate, rodent, guinea pig, dog, cat, rabbit, and pig.

62. A method for identifying an agent that inhibits plaque component induced neurotoxicity of a mononuclear phagocyte comprising:

contacting a mononuclear phagocyte with a plaque component coupled to a solid support to stimulate plaque component induced neurotoxicity of a mononuclear phagocyte together with an agent suspected of inhibiting said plaque component induced neurotoxicity of said mononuclear phagocyte;

measuring plaque component induced neurotoxicity of said mononuclear phagocyte; and

comparing said measured plaque component induced neurotoxicity of said mononuclear phagocyte to that of a measured control,

wherein reduction of plaque component induced neurotoxicity compared to that of said control results in the identification of said inhibitory agent.

63. A method for identifying an agent that inhibits plaque component induced neurotoxicity of a mononuclear phagocyte comprising:

contacting a mononuclear phagocyte in a mammalian brain with a plaque component to stimulate plaque component induced neurotoxicity together with an agent suspected of inhibiting said plaque component induced neurotoxicity;

measuring plaque component induced neurotoxicity of said mononuclear phagocyte; and

comparing said measured plaque component induced neurotoxicity to that of a measured control,

wherein reduction of plaque component induced neurotoxicity compared to that of said control results in the identification of said inhibitory agent.

64. The method of claim 63 wherein said step of measuring is selected from the group consisting of observing a loss of metabolic function, release of intracellular material, penetration of impermeant dyes, and reduction of cell number of neurons.

65. The method of claim 63 wherein said plaque component or said mononuclear phagocyte is labeled.

66. The method of claim 65 wherein said label is selected from the group consisting of ^{32}P, ^{125}I, ^{14}C, ^{3}H, ^{35}S, biotin, fluorescein, rhodamine, peroxidase, and antibody labeling.

67. The method of claim 63 wherein said step of measuring comprises imaging said plaque component induced mononuclear phagocyte neurotoxicity and observing a signal resulting from said imaging.

68. The method of claim 47 wherein said step of measuring comprises detecting amplified nucleic acids from said plaque component induced mononuclear phagocyte neurotoxicity.

69. The method of claim 47 wherein said mononuclear phagocyte is selected from the group consisting of a microglial cell, a monocyte, a macrophage, and a microglial precursor cell, monocyte precursor cell, a macrophage precursor cell, microglial-like cell, monocyte-like cell, and a macrophage-like cell.

70. A method for identifying an agent that inhibits the toxic effect on a neuron of a neurotoxin from a plaque component activated mononuclear phagocyte comprising:

contacting a neuron with a neurotoxin from a plaque component activated mononuclear phagocyte, or a plaque component induced neurotoxic mononuclear phagocyte to stimulate neuronal damage together with an agent suspected of inhibiting the effect of said neurotoxin;

measuring neuron function; and

comparing said measured neuron function to that of a measured control,

wherein an increase in neuron function compared to that of said control results in the identification of said inhibitory agent.

71. The method of claim 70 wherein said agent is a neurotoxin blocker.

72. The method of claim 70 wherein said plaque component is selected from the group consisting of β amyloid and active fragments thereof, α-antichymotrypsin, apolipoprotein A, apolipoprotein E, glycoproteins, heparan sulfate, and proteases.

73. The method of claim 70 wherein said plaque component is β amyloid and said agent is a neurotoxin blocker.

74. The method of claim 70 wherein said neuron or said neurotoxin is adhered to a solid support.

75. The method of claim 74 wherein said solid support is selected from a microsphere, liposome, sepharose, and sephadex.

76. The method of claim 74 wherein said solid support is a microsphere and said neurotoxin is adhered to said microsphere.

77. The method of claim 70 wherein said neurotoxin is adhered to a solid support and is infused into a mammalian brain selected from the group consisting of primate, rodent, guinea pig, dog, cat, rabbit, and pig.

78. The method of claim 70 wherein said step of measuring comprises observing disruption of normal neuron metabolism.

79. The method of claim 78 wherein said normal cell metabolism is selected from the group consisting of metabolism of glucose, the production of ATP, maintenance of ion gradients across a cell membrane, protein synthesis, nucleic acid synthesis, and mitochondrial respiration.

80. The method of claim 70 wherein said step of measuring comprises detecting amplified nucleic acids from said neuron.

81. The method of claim 70 wherein said plaque component is selected from a patient suspected of having a disease selected from the group consisting of Alzheimer Disease, hereditary hemorrhage with amyloidosis-Dutch type, cerebral amyloid angiopathy, cerebral amyloid angiopathy, Down's syndrome, spongiform encephalopathy, Creutzfeld-Jakob disease, HIV, Parkinson's disease, multiple sclerosis, amyotrophic lateral sclerosis, stroke, and trauma.

82. The method of claim 70 wherein said neurotoxin or said neuron is adhered to a solid support and is infused into a mammalian brain selected from the group consisting of primate, rodent, guinea pig, dog, cat, rabbit, and pig.

83. A method for identifying an agent that inhibits the effect of a neurotoxin from a plaque component activated a mononuclear phagocyte on neurons comprising:

contacting a neuron with a neurotoxin coupled to a solid support from a plaque component activated mononuclear phagocyte to stimulate neuronal damage together with an agent suspected of inhibiting said neurotoxin;

measuring neuron function; and

comparing said measured neuron function to that of a measured control,

wherein an increase in neuron function compared to that of said control results in the identification of said inhibitory agent.

84. A method for identifying an agent that inhibits the toxic effect on neurons of a neurotoxin from a plaque component activated a mononuclear phagocyte comprising:

contacting a neuron in a mammalian brain with a neurotoxin coupled to a solid support from a plaque component activated mononuclear phagocyte to stimulate neuronal damage together with an agent suspected of inhibiting said neurotoxin;

measuring neuron function; and

comparing said measured neuron function to that of a measured control,

wherein an increase in neuron function compared to that of said control results in the identification of said inhibitory agent.

85. The method of claim 84 wherein said step of measuring comprises observing disruption of normal neuron metabolism.

86. The method of claim 85 wherein said normal cell metabolism is selected from the group consisting of metabolism of glucose, the production of ATP, maintenance of ion gradients across a cell membrane, protein synthesis, nucleic acid synthesis, and mitochondrial respiration.

87. The method of claim 84 wherein said step of measuring comprises detecting amplified nucleic acids from said neuron.

88. The method of claim 84 wherein said mononuclear phagocyte is selected from the group consisting of a microglial cell, a monocyte, a macrophage, and a microglial precursor cell, monocyte precursor cell, a macrophage precursor cell, microglial-like cell, monocyte-like cell, and macrophage-like cell.

89. A method for identifying an agent that inhibits the effect of a neurotoxin from a plaque component activated mononuclear phagocyte on a neuron comprising:

contacting a mononuclear phagocyte with a plaque component to stimulate mononuclear phagocyte-plaque component complex formation together with an agent suspected of inhibiting said complex formation;

measuring of mononuclear phagocyte-plaque component complex formation;

comparing said measured mononuclear phagocyte-plaque component complex formation to that of a measured control,

wherein a reduction of mononuclear phagocyte-plaque component complex formation compared to that of said control results in the identification of said inhibitory agent, and isolating a mononuclear phagocyte from an inhibited mononuclear phagocyte-plaque component complex formation,

contacting a mononuclear phagocyte from an inhibited mononuclear phagocyte-plaque component complex with a plaque component to stimulate plaque component activation of said mononuclear phagocyte together with an agent suspected of inhibiting said activation;

measuring plaque component activation of said mononuclear phagocyte;

comparing said plaque component activation of said measured mononuclear phagocyte to that of a measured control,

wherein reduction of plaque component activation of said mononuclear phagocyte compared to that of said control results in the identification of said inhibitory agent, and isolating a mononuclear phagocyte which is plaque component activation inhibited,

contacting a mononuclear phagocyte which is plaque component activation inhibited with a plaque component to stimulate plaque component induced neurotoxicity

together with an agent suspected of inhibiting said plaque component induced neurotoxicity;

measuring plaque component induced neurotoxicity,

comparing said measured plaque component induced neurotoxicity to that of a measured control,

wherein a reduction of plaque component induced neurotoxicity compared to said control results in the identification of said inhibitory agent, and isolating a plaque component induced neurotoxic mononuclear phagocyte,

contacting a neuron with a neurotoxin from a plaque component activated mononuclear phagocyte, or a plaque component induced neurotoxic mononuclear phagocyte to stimulate neuronal damage together with an agent suspected of inhibiting said neurotoxin;

measuring neuron function;

comparing said measured neuron function to that of a measured control,

wherein an increase in neuron function compared to that of said control results in the identification of said inhibitory agent.

90. A method for identifying an agent that inhibits plaque component induced neurotoxicity of a mononuclear phagocyte comprising performing the method of claim 1 and isolating a mononuclear phagocyte from an inhibited mononuclear phagocyte-plaque component complex formation, performing the method of claim 24 and isolating a mononuclear phagocyte which is plaque component activation inhibited, and performing the method of claim 47 thereby identifying said inhibitory agent.

91. An agent identified by the method of claim 1, wherein said agent comprises an HHQK sequence.

92. An agent of claim 91 having mononuclear phagocyte-plaque component complex formation inhibitory activity.

93. A composition comprising an HHQK-like molecule that inhibits a mononuclear phagocyte-plaque component complex in a pharmaceutically acceptable carrier.

94. An agent identified by the method of claim 47, wherein said agent comprises a chloroquine.

95. An agent of claim 94 having neurotoxic mononuclear phagocyte inactivator activity.

96. A composition comprising a chloroquine that inhibits a neurotoxicity of a mononuclear phagocyte induced by a plaque component complex in a pharmaceutically acceptable carrier.

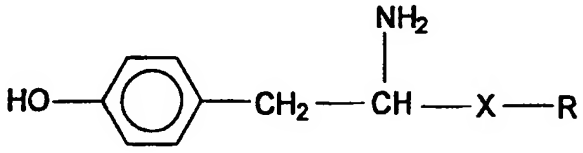
97. An agent identified by the method of claim 70, wherein said agent comprises a tyramine.

98. An agent of claim 97 having neurotoxin blocking activity.

99. A composition comprising a tyramine that inhibits the effects of neurotoxins on neurons in a pharmaceutically acceptable carrier.

100. A method of inhibiting the injury, destruction or death of neurons in a patient comprising administering to the patient a tyramine compound or a physiologically acceptable salt thereof.

101. The method of claim 100 wherein the tyramine compound has the formula:



wherein X is an ester group, an amide group, an ether group, an alkyl group having from 1 to about 20 carbon atoms or an alkyl halide group having from 1 to about 20 carbon atoms; and R is a linear, branched or cyclic, saturated or unsaturated hydrocarbon group having from 3 to about 50 carbon atoms that is optionally interrupted with one or more of an oxygen atom, a nitrogen atom, a sulfur atom or a halide atom.

102. The method of claim 101 wherein X is an ester group, an amide group or an ether group.

103. The method of claim 101 wherein R is a t-butyl group, an allyl group, a benzyl group or a naphthyl group.

104. The method of claim 101 wherein R is an indole group, a pyrrole group, an imidazole group, a furan group, a tosyl group, a thiophene group, a piperidine group, a phenothiazine group, a benzodiazepam group or a muscarine group.

105. The method of claim 100 wherein the tyramine compound is L-tyrosine t-butyl ester, L-tyrosine allyl ester, L-tyrosine benzyl ester or L-tyrosine β-naphthylamide.

106. The method of claim 100 wherein the tyramine compound has the formula:

$$Y\text{-}CH_2\text{-}CH(NH_2)\text{-}X\text{-}R$$

wherein Y is an indole group, a pyrrole group, an imidazole group, a tosyl group, a furan group or a thiophene group; X is an ester group, an amide group, an ether group, an alkyl group having from 1 to about 20 carbon atoms or an alkyl halide group having from 1 to about 20 carbon atoms; and R is a linear, branched or cyclic, saturated or unsaturated hydrocarbon group having from 3 to about 50 carbon atoms that is optionally interrupted with one or more of an oxygen atom, a nitrogen atom, a sulfur atom or a halide atom.

107. A method of inhibiting the injury, destruction or death of neurons in a patient comprising administering to the patient a compound that inhibits the toxic effects of a neurotoxin.

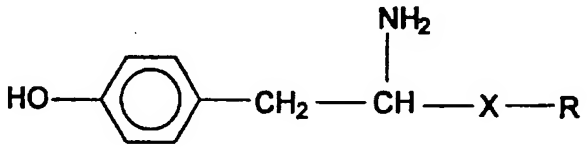
108. The method of claim 107 wherein a plaque component affects release of the neurotoxin from a mononuclear phagocyte.

109. The method of claim 108 wherein at least one neurotoxin is a phenolic amine.

110. The method of claim 108 wherein the compound is a tyramine compound.

111. The method of claim 108 wherein the compound is tyramine.

112. The method of claim 110 wherein the tyramine compound is of the formula:



wherein X is a linking group selected from the group consisting of an ester group, an amide group, an ether group, an alkyl group having from 1 to about 20 carbon atoms or an alkyl halide group having from 1 to about 20 carbon atoms; and R is a linear, branched or cyclic, saturated or unsaturated hydrocarbon group having from 3 to about 50 carbon atoms that is optionally interrupted with one or more of an oxygen atom, a nitrogen atom, a sulfur atom or a halide atom.

113. The method of claim 112 wherein R is a t-butyl group, an allyl group, a benzyl group or a naphthyl group.

114. The method of claim 112 wherein R is an indole group, a pyrrole group, an imidazole group, a furan group, a tosyl group, a thiophene group, a piperidine group, a phenothiazine group, a benzodiazepam group or a muscarine group.

115. The method of claim 112 wherein X is an ester group, an amide group or an ether group.

116. The method of claim 112 wherein the compound is L-tyrosine t-butyl ester, L-tyrosine allyl ester, L-tyrosine benzyl ester or L-tyrosine β-naphthylamide.

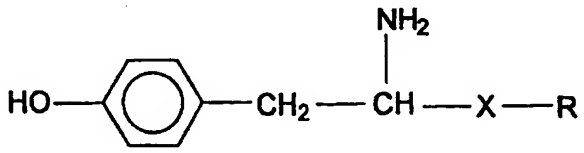
117. The method of claim 110 wherein the tyramine compound is of the formula:

$$Y\text{-}CH_2\text{-}CH(NH_2)\text{-}X\text{-}R$$

wherein Y is an indole group, a pyrrole group, an imidazole group, a tosyl group, a furan group or a thiophene group; X is an ester group, an amide group, an ether group, an alkyl group having from 1 to about 20 carbon atoms or an alkyl halide group having from 1 to about 20 carbon atoms; and R is a linear, branched or cyclic, saturated or unsaturated hydrocarbon group having from 3 to about 50 carbon atoms that is optionally interrupted with one or more of an oxygen atom, a nitrogen atom, a sulfur atom or a halide atom.

118. A method of treating a neurodegenerative disease or disorder in a patient comprising administering to the patient a tyramine compound or a physiologically acceptable salt thereof.

119. The method of claim 118 wherein the tyramine compound is of the formula:



wherein X is an ester group, an amide group, an ether group, an alkyl group having from 1 to about 20 carbon atoms or an alkyl halide group having from 1 to about 20 carbon atoms; and R is a linear, branched or cyclic, saturated or unsaturated hydrocarbon group having from 3 to about 50 carbon atoms that is optionally interrupted with one or more of an oxygen atom, a nitrogen atom, a sulfur atom or a halide atom.

120. The method of claim 119 wherein X is an ester group, an amide group or an ether group.

121. The method of claim 119 wherein R is a t-butyl group, an allyl group, a benzyl group or a naphthyl group.

122. The method of claim 119 wherein R is an indole group, a pyrrole group, an imidazole group, a furan group, a tosyl group, a thiophene group, a piperidine group, a phenothiazine group, a benzodiazepam group or a muscarine group.

123. The method of claim 119 wherein the tyramine compound is L-tyrosine t-butyl ester, L-tyrosine allyl ester, L-tyrosine benzyl ester or L-tyrosine β-naphthylamide.

124. The method of claim 118 wherein the tyramine compound is of the formula:

$$Y\text{-}CH_2\text{-}CH(NH_2)\text{-}X\text{-}R$$

wherein Y is an indole group, a pyrrole group, an imidazole group, a tosyl group, a furan group or a thiophene group; X is an ester group, an amide group, an ether group, an alkyl group having from 1 to about 20 carbon atoms or an alkyl halide group having from 1 to about 20 carbon atoms; and R is a linear, branched or cyclic, saturated or unsaturated hydrocarbon group having from 3 to about 50 carbon atoms that is optionally interrupted with one or more of an oxygen atom, a nitrogen atom, a sulfur atom or a halide atom.

125. A method of treating neurodegenerative diseases or disorders in a patient comprising administering to the patient a compound that inhibits the toxic effects of a neurotoxin.

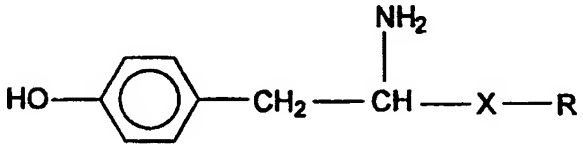
126. The method of claim 125 wherein a plaque component affects release of the neurotoxin from a mononuclear phagocyte.

127. The method of claim 126 wherein at least one neurotoxin is a phenolic amine.

128. The method of claim 125 wherein the compound is a tyramine compound.

129. The method of claim 125 wherein the compound is tyramine.

130. The method of claim 125 wherein the tyramine compound is of the formula:



wherein X is an ester group, an amide group, an ether group, an alkyl group having from 1 to about 20 carbon atoms or an alkyl halide group having from 1 to about 20 carbon atoms; and R is a linear, branched or cyclic, saturated or unsaturated hydrocarbon group having from 3 to about 50 carbon atoms that is optionally interrupted with one or more of an oxygen atom, a nitrogen atom, a sulfur atom or a halide atom.

131. The method of claim 130 wherein R is a t-butyl group, an allyl group, a benzyl group or a naphthyl group.

132. The method of claim 130 wherein R is an indole group, a pyrrole group, an imidazole group, a furan group, a tosyl group, a thiophene group, a piperidine group, a phenothiazine group, a benzodiazepam group or a muscarine group.

133. The method of claim 130 wherein X is an ester group, an amide group or an ether group.

134. The method of claim 130 wherein the compound is L-tyrosine t-butyl ester, L-tyrosine allyl ester, L-tyrosine benzyl ester or L-tyrosine β-naphthylamide.

135. The method of claim 125 wherein the tyramine compound is of the formula:

$$Y\text{-}CH_2\text{-}CH(NH_2)\text{-}X\text{-}R$$

wherein Y is an indole group, a pyrrole group, an imidazole group, a tosyl group, a furan group or a thiophene group; X is an ester group, an amide group, an ether group, an alkyl group having from 1 to about 20 carbon atoms or an alkyl halide group having from 1 to about 20 carbon atoms; and R is a linear, branched or cyclic, saturated or unsaturated hydrocarbon group having from 3 to about 50 carbon atoms that is optionally interrupted with one or more of an oxygen atom, a nitrogen atom, a sulfur atom or a halide atom.

136. The method of claim 125 wherein the neurodegenerative disease or disorder is Alzheimer's disease, HIV-1 infection, AIDS dementia, amyotrophic lateral sclerosis, stroke, trauma, hereditary hemorrhage with amyloidosis-Dutch type, cerebral amyloid angiopathy, Creutzfeld-Jakob disease, Parkinson's disease or multiple sclerosis.

137. A method of identifying an agent that inhibits the toxic effects of a neurotoxin comprising:

contacting a neuron with a neurotoxic amount of said neurotoxin and an agent suspected of inhibiting said toxic effect,

comparing the inhibition of said agent to a measured control obtained by contacting a neuron with a neurotoxic amount of a neurotoxin and a tyramine compound.

138. A method of identifying an agent that inhibits the toxic effects of a neurotoxin comprising:

contacting a neuron with a neurotoxic amount of said neurotoxin, at least one agent suspected of inhibiting said toxic effect, and a tyramine compound;

comparing the inhibition of said agent with the inhibition of said tyramine compound.

139. The method of claim 138 wherein said comparison is of competitive binding.

140. The method of claim 138 wherein said tyramine compound is detectably labeled.

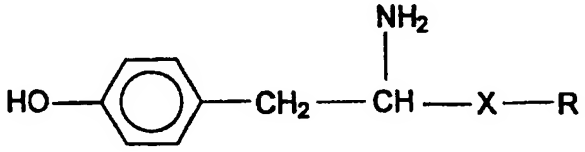
141. A method of assaying a neurotoxin in a patient comprising:

contacting a neuron with a sample from said patient and a tyramine compound;

measuring the inhibition of said tyramine compound in said sample; and

comparing the inhibition of said tyramine compound in said sample to a measured control.

142. A unit dosage of a tyramine compound in a pharmaceutically acceptable carrier or diluent, wherein the tyramine compound is of the formula:



wherein X is an ester group, an amide group, an ether group, an alkyl group having from 1 to about 20 carbon atoms or an alkyl halide group having from 1 to about 20 carbon atoms; and R is a linear, branched or cyclic, saturated or unsaturated hydrocarbon group having from 3 to about 50 carbon atoms.

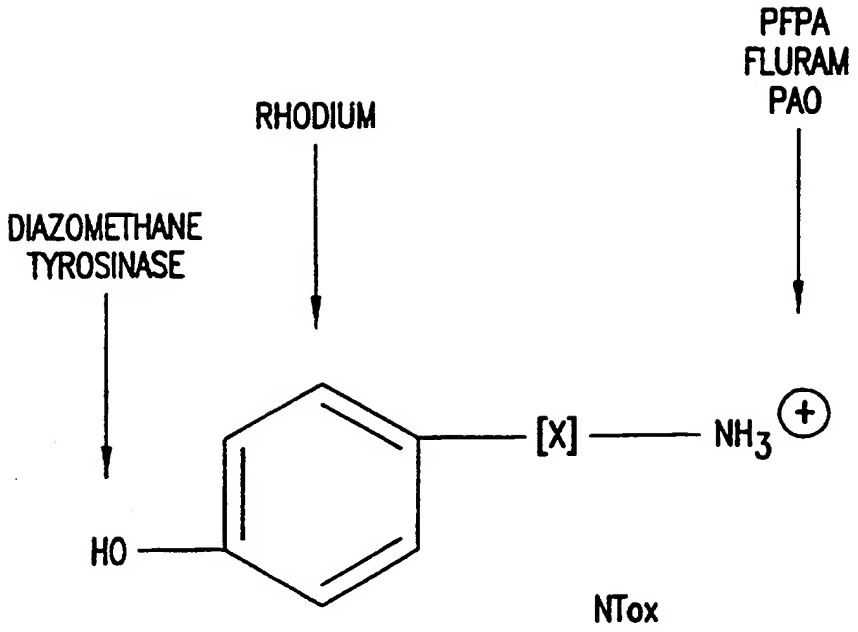

PFPA
FLURAM
PAO
RHODIUM
DIAZOMETHANE
TYROSINASE
[X]
NH3
HO
NTox

FIG.1

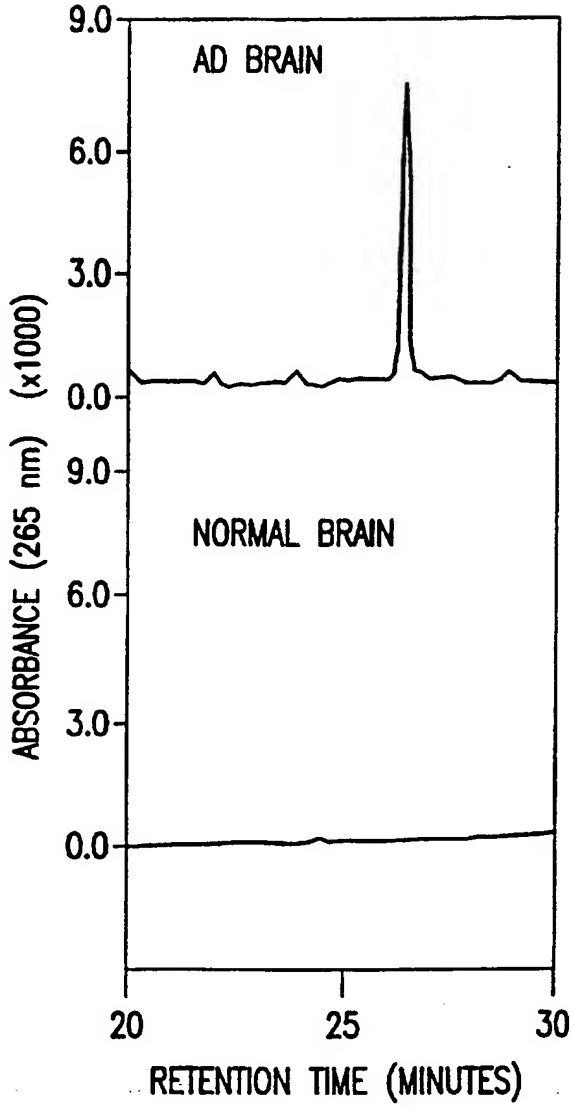

AD BRAIN
NORMAL BRAIN
ABSORBANCE (265 nm) (x1000)
9.0
6.0
3.0
0.0
9.0
6.0
3.0
0.0
20
25
30
RETENTION TIME (MINUTES)

FIG.2A

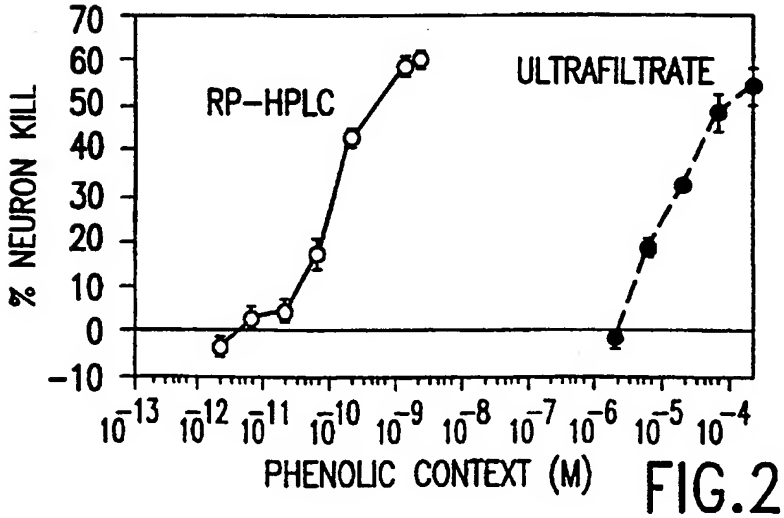

RP-HPLC
ULTRAFILTRATE
% NEURON KILL
70
60
50
40
30
20
10
0
-10
10^{-13} 10^{-12} 10^{-11} 10^{-10} 10^{-9} 10^{-8} 10^{-7} 10^{-6} 10^{-5} 10^{-4}
PHENOLIC CONTEXT (M)

FIG.2B

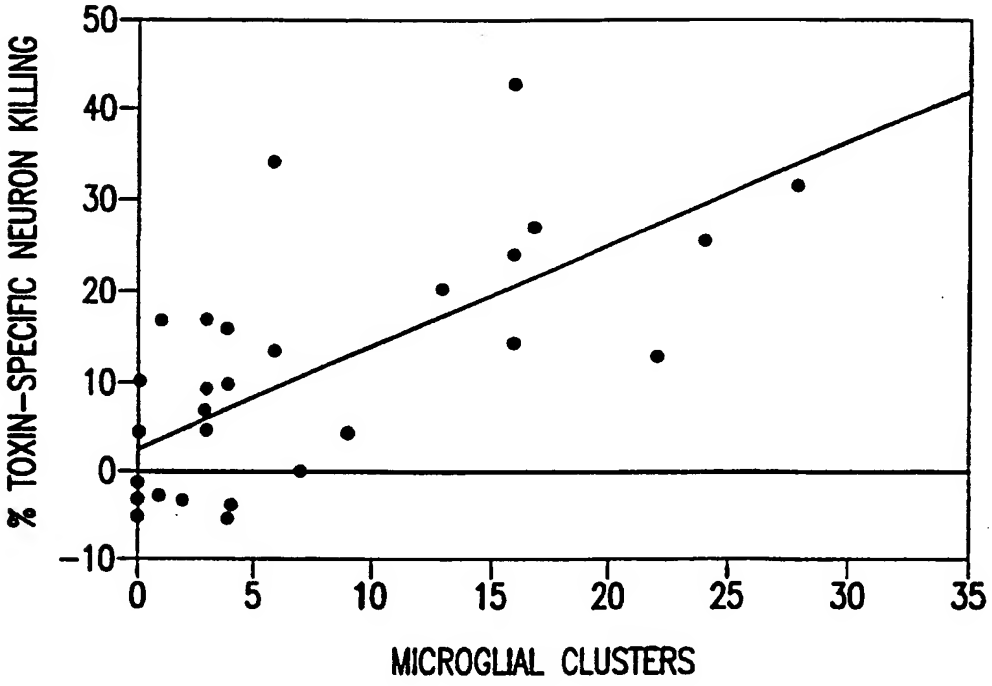

% TOXIN-SPECIFIC NEURON KILLING
50
40
30
20
10
0
-10
0
5
10
15
20
25
30
35
MICROGLIAL CLUSTERS

FIG.3

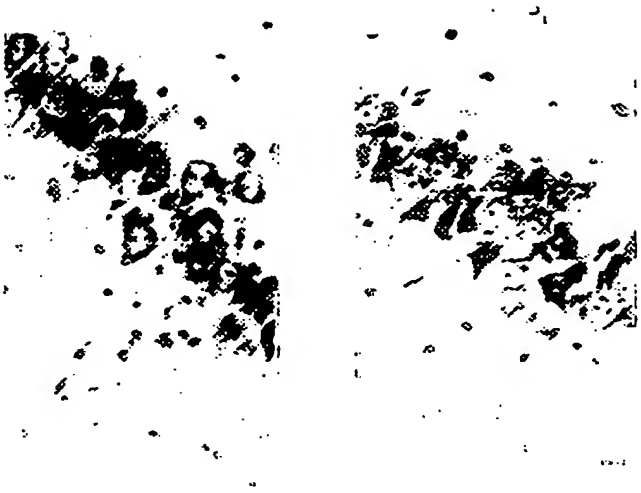


FIG.4A FIG.4B

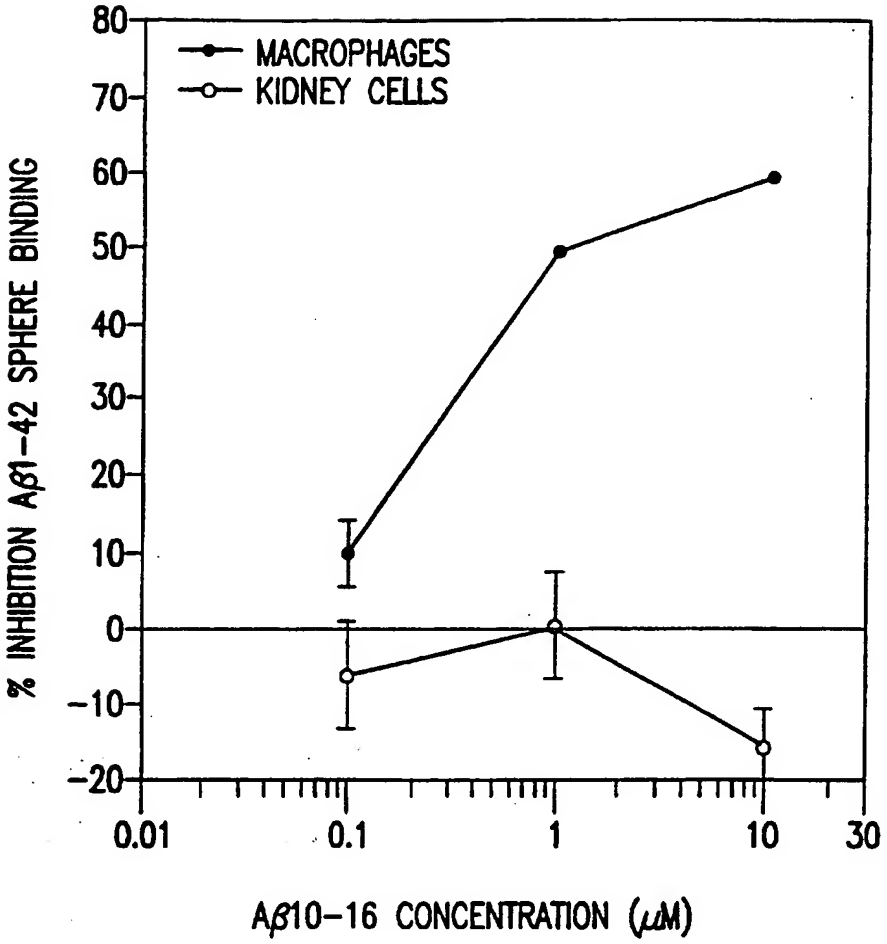

MACROPHAGES
KIDNEY CELLS
% INHIBITION Aβ1-42 SPHERE BINDING
80
70
60
50
40
30
20
10
0
-10
-20
0.01
0.1
1
10
30
Aβ10-16 CONCENTRATION (μM)

FIG.5

FIG.6A

FIG.6B

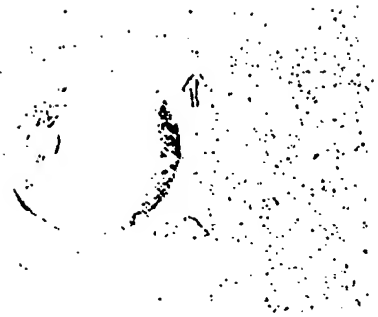


FIG.7A

FIG.7B

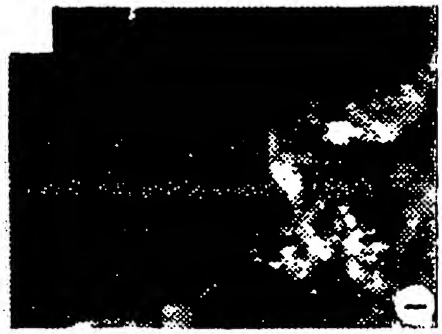


FIG.7C

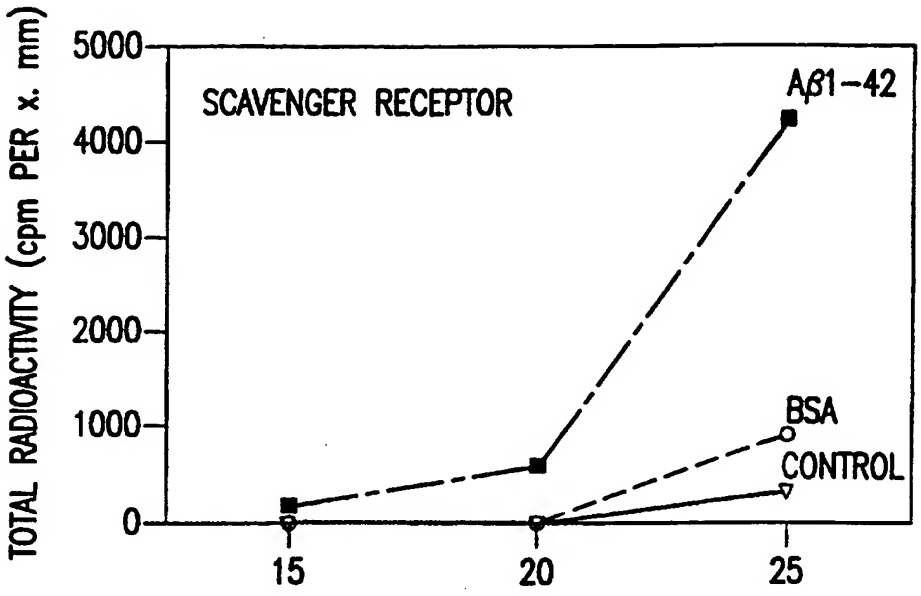

TOTAL RADIOACTIVITY (cpm PER x. mm)
5000
4000
3000
2000
1000
0
SCAVENGER RECEPTOR
Aβ1-42
BSA
CONTROL
15
20
25

FIG.8A

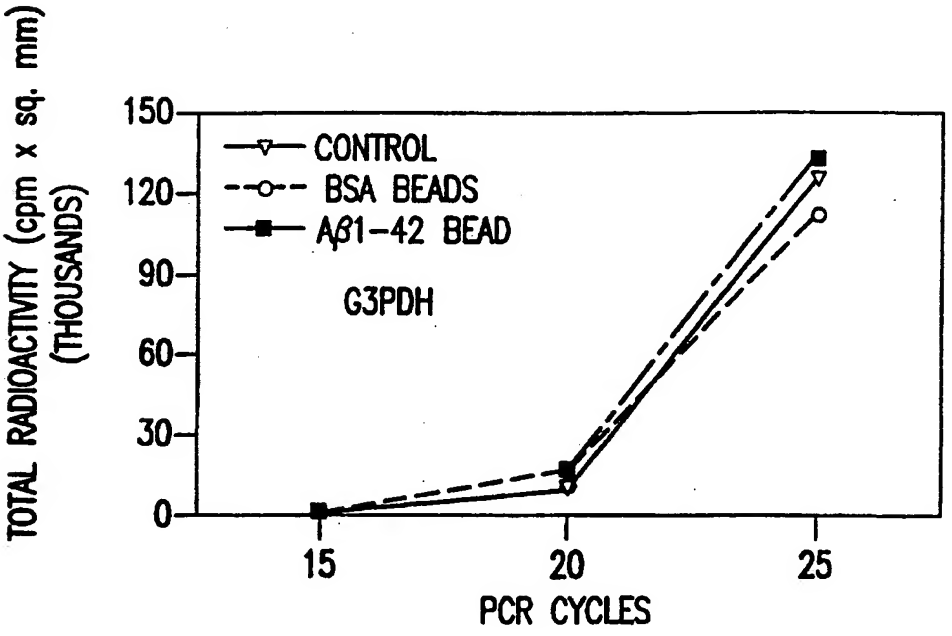

TOTAL RADIOACTIVITY (cpm x sq. mm)
(THOUSANDS)
150
120
90
60
30
0
CONTROL
BSA BEADS
Aβ1-42 BEAD
G3PDH
15
20
25
PCR CYCLES

FIG.8B

FIG.9A

FIG.9B

FIG.9C

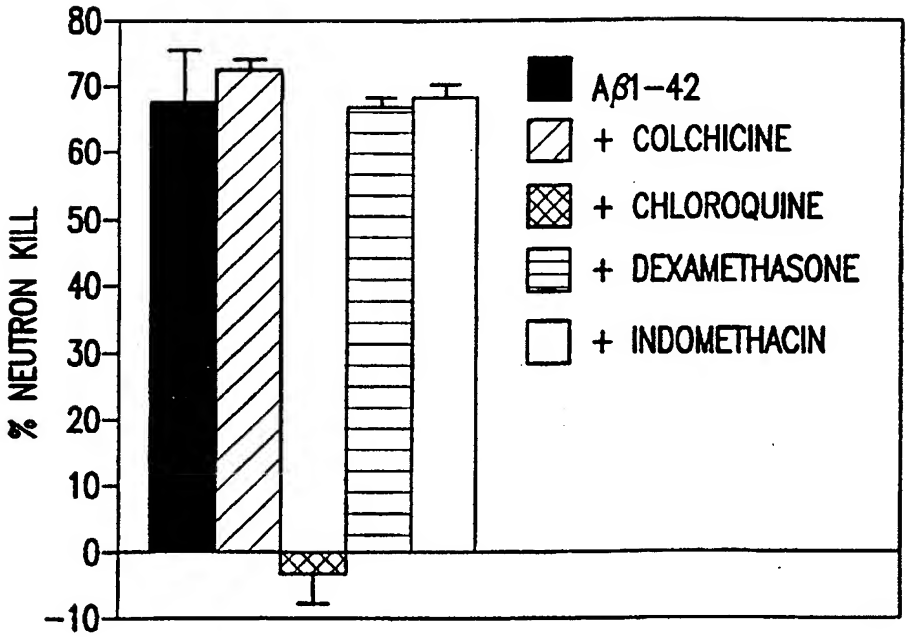

80
70
60
50
40
30
20
10
0
-10
% NEUTRON KILL
Aβ1-42
+ COLCHICINE
+ CHLOROQUINE
+ DEXAMETHASONE
+ INDOMETHACIN

FIG.10

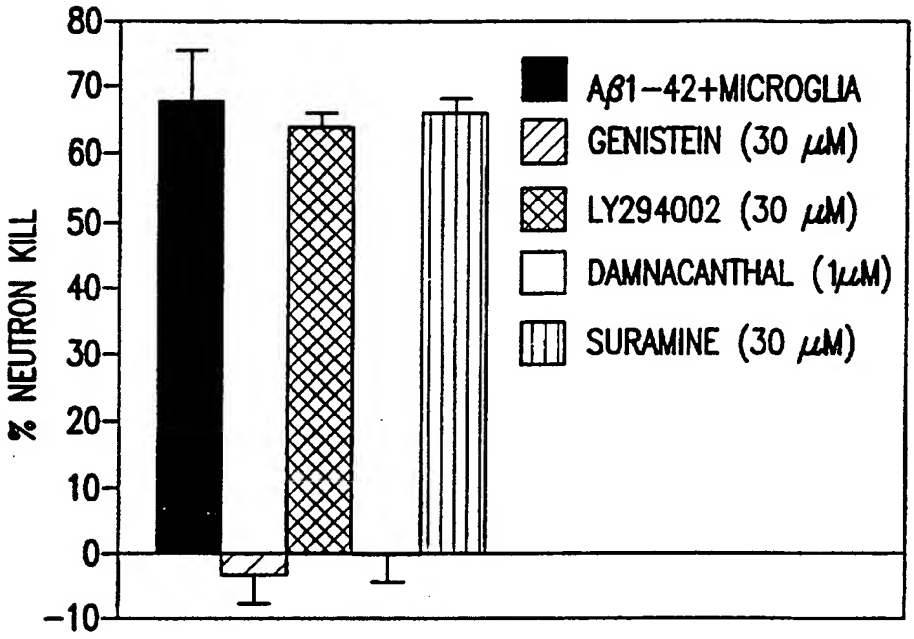
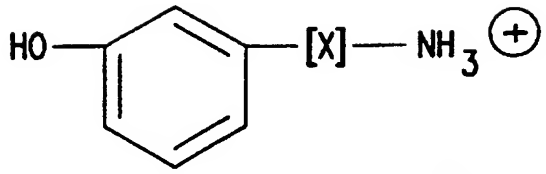
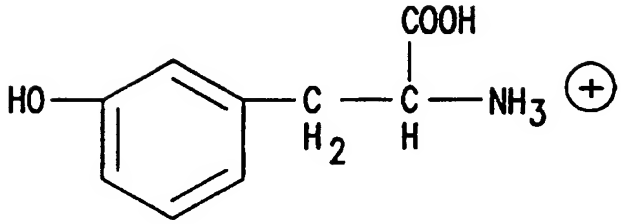

80
70
60
50
40
30
20
10
0
-10
% NEUTRON KILL
Aβ1-42+MICROGLIA
GENISTEIN (30 μM)
LY294002 (30 μM)
DAMNACANTHAL (1μM)
SURAMINE (30 μM)

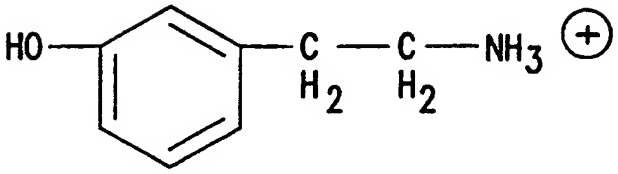
FIG.11



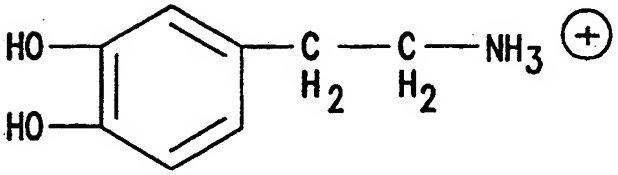
NTox



TYROSINE



TYRAMINE



DOPAMINE

FIG.12

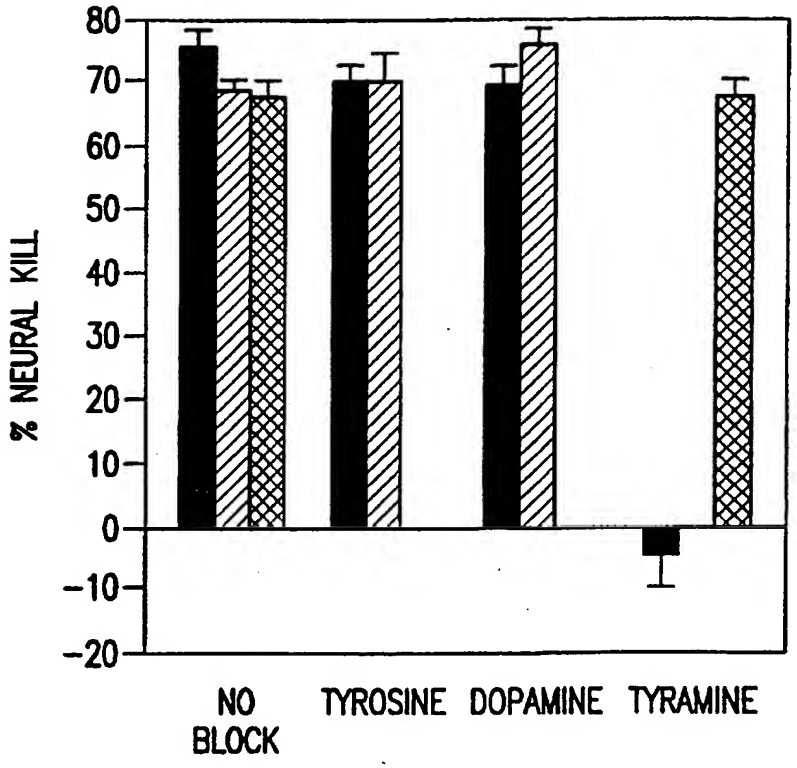

80
70
60
50
40
30
20
10
0
−10
−20
% NEURAL KILL
NO BLOCK
TYROSINE
DOPAMINE
TYRAMINE
Aβ1−42 + MICROGLIA
NTox
QUIN 100 μM

FIG.13

FIG.14A

FIG.14B

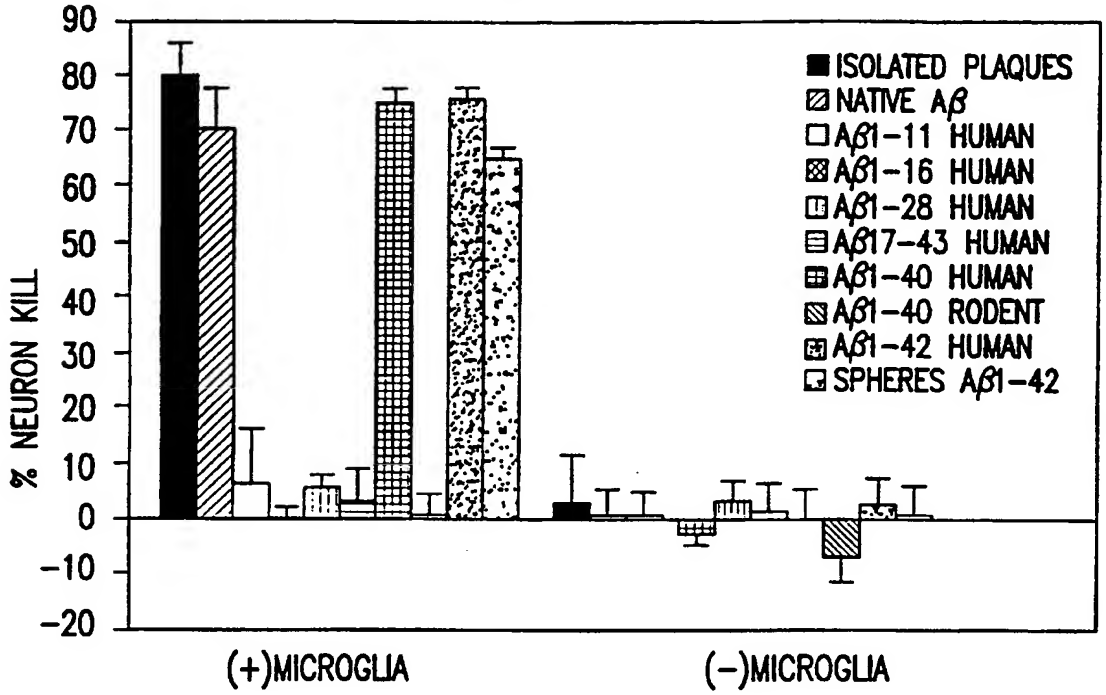


FIG.14C

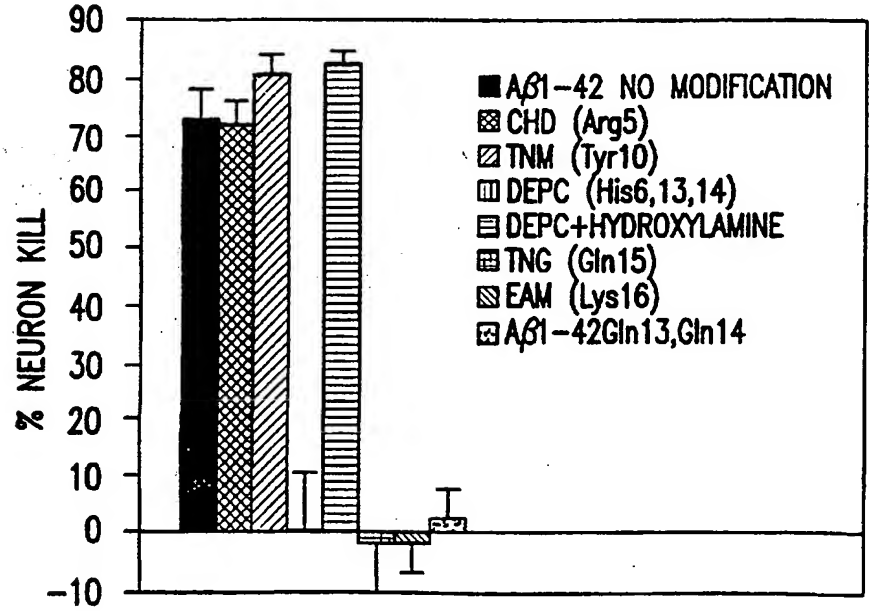


FIG.14D

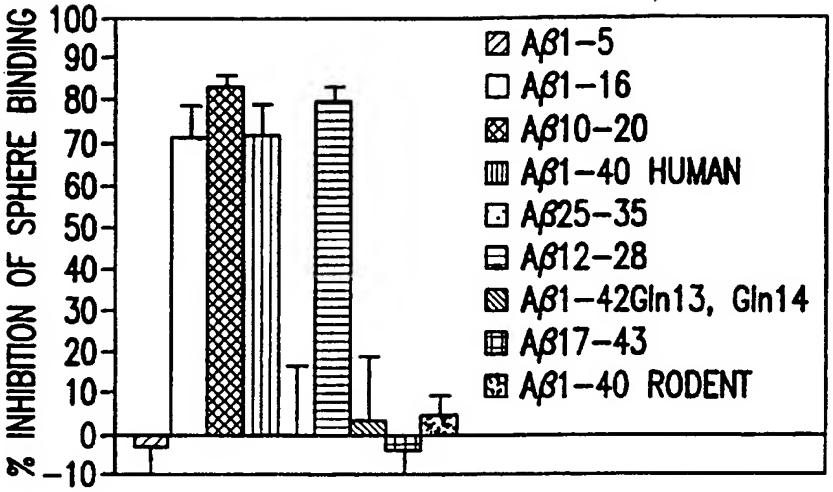


FIG.15A

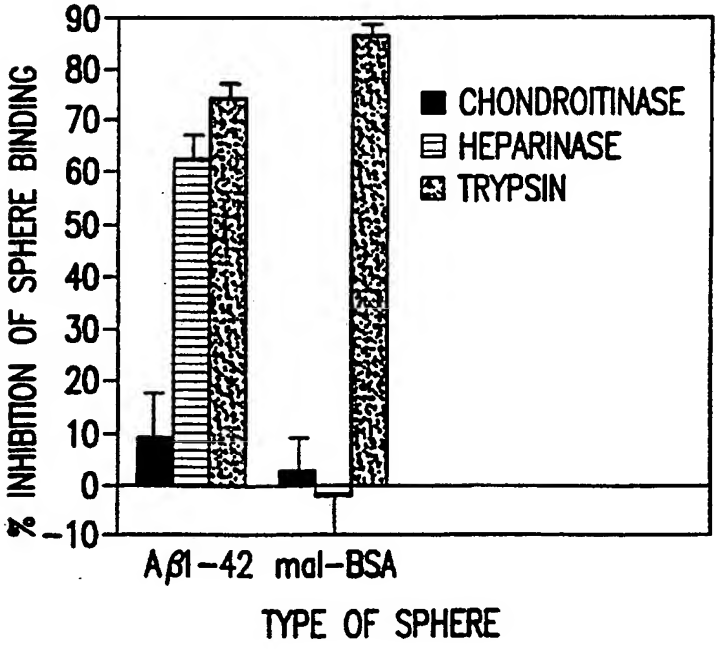


FIG.15B

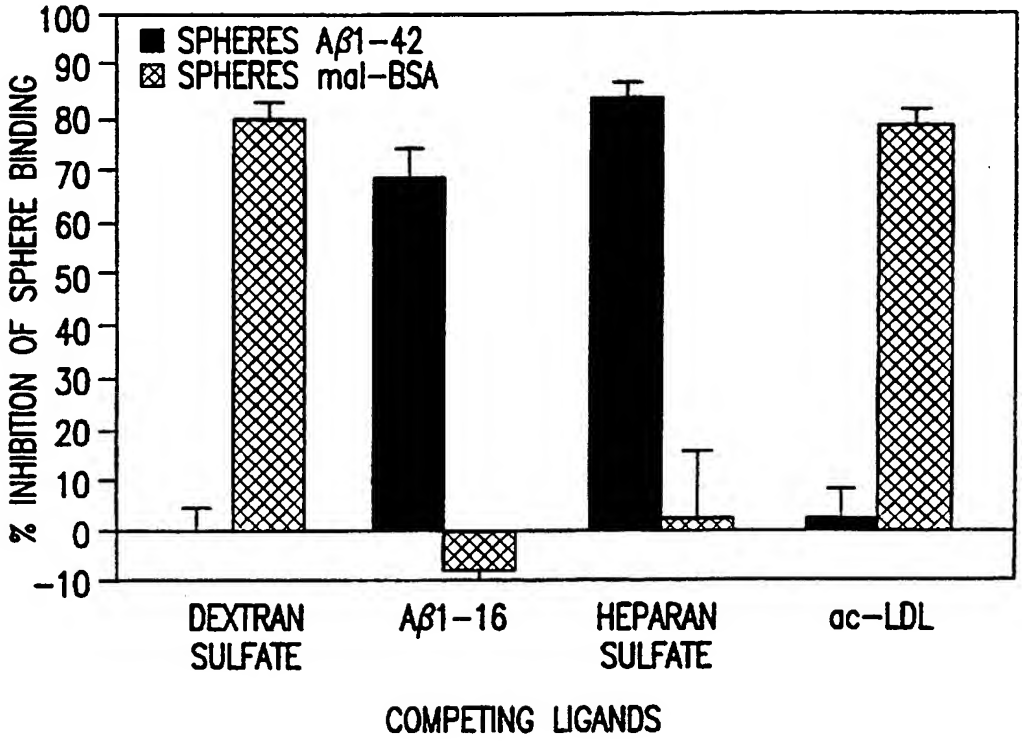


FIG.15C

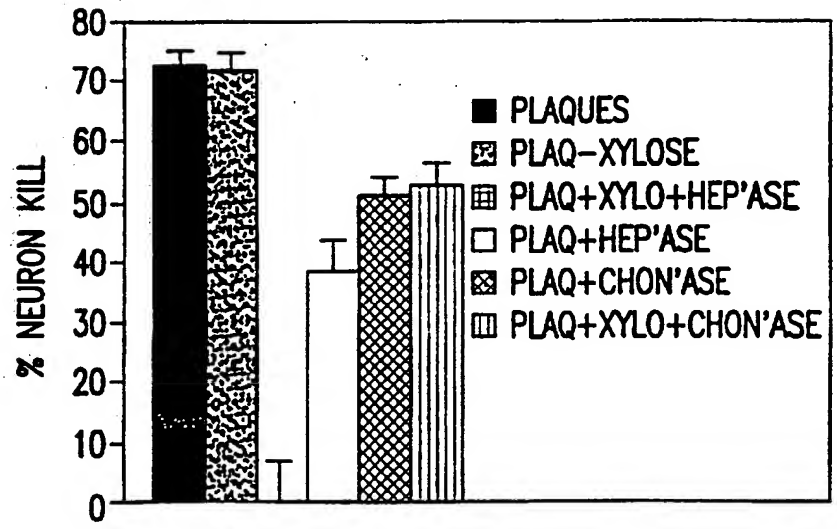


FIG.15D

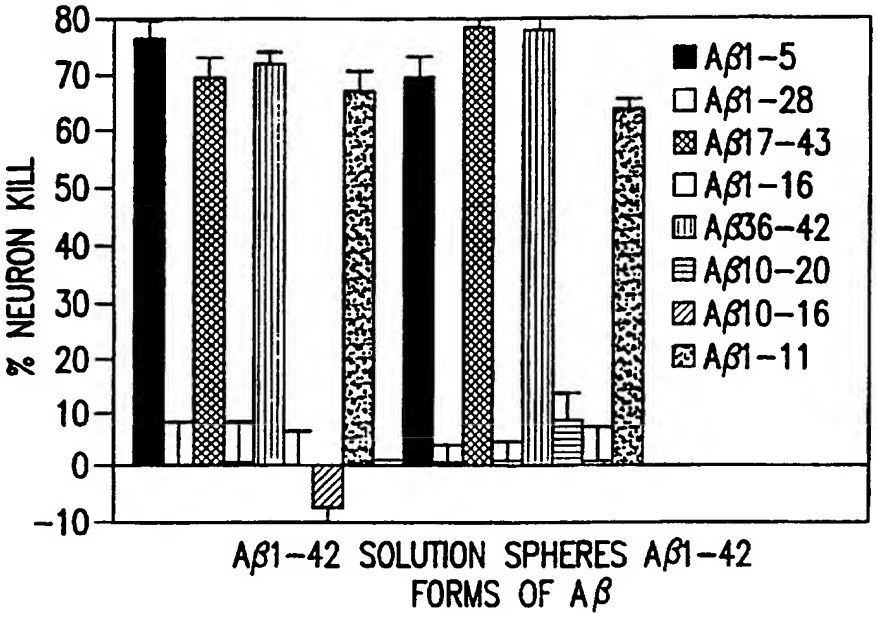


FIG.16A

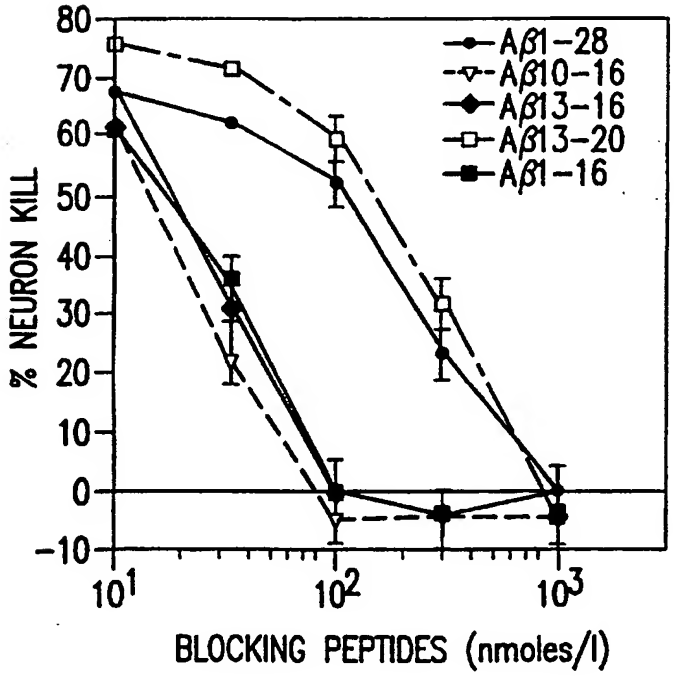


FIG.16B

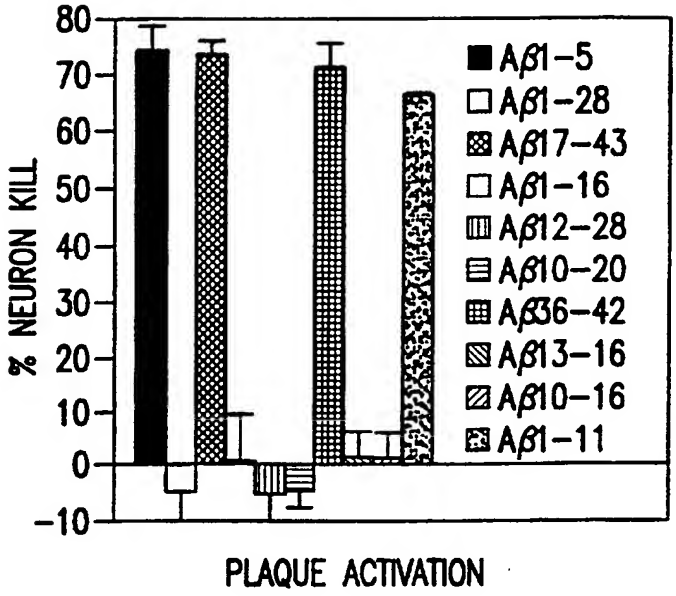

80
70
60
50
40
30
20
10
0
-10
% NEURON KILL
Aβ1-5
Aβ1-28
Aβ17-43
Aβ1-16
Aβ12-28
Aβ10-20
Aβ36-42
Aβ13-16
Aβ10-16
Aβ1-11
PLAQUE ACTIVATION

FIG.16C

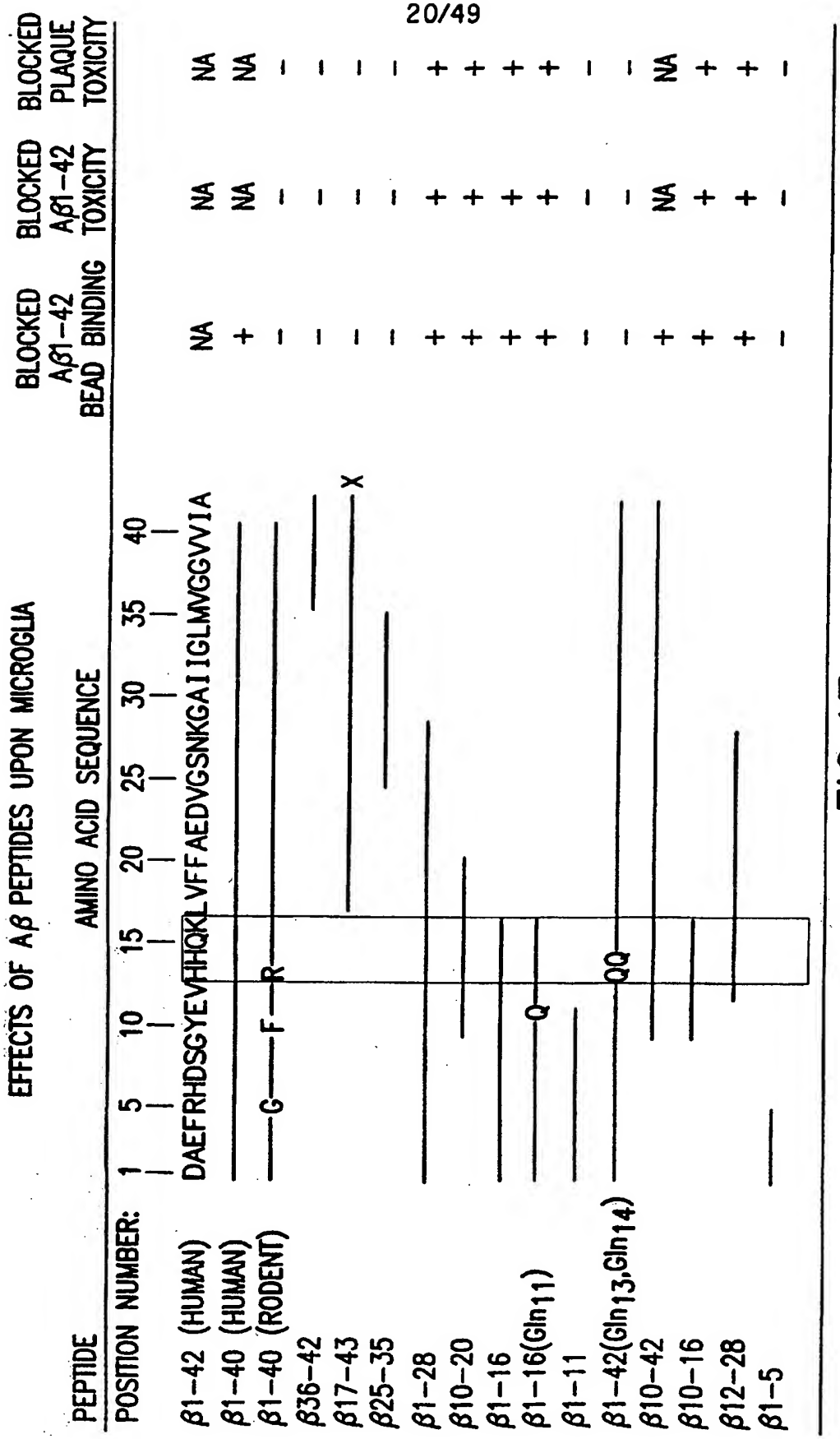

EFFECTS OF Aβ PEPTIDES UPON MICROGLIA
AMINO ACID SEQUENCE
PEPTIDE
POSITION NUMBER: 1 5 10 15 20 25 30 35 40
DAEFRHDSGYEVHHQKLVFFAEDVGSNKGAIIGLMVGGVVIA
BLOCKED Aβ1-42 BEAD BINDING
BLOCKED Aβ1-42 TOXICITY
BLOCKED PLAQUE TOXICITY
β1-42 (HUMAN) NA NA NA
β1-40 (HUMAN) + NA NA
β1-40 (RODENT) G F R - - -
β36-42 - - -
β17-43 X - - -
β25-35 - - -
β1-28 + + +
β10-20 + + +
β1-16 + + +
β1-16(Gln11) Q + + +
β1-11 - - -
β1-42(Gln13,Gln14) QQ - - -
β10-42 + NA NA
β10-16 + + +
β12-28 + + +
β1-5 - - -

FIG.17

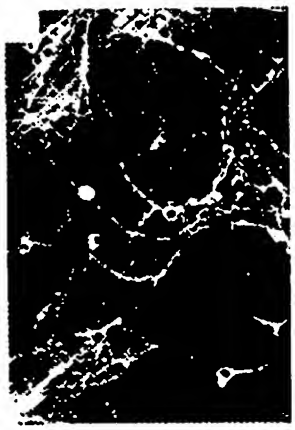


FIG.18A

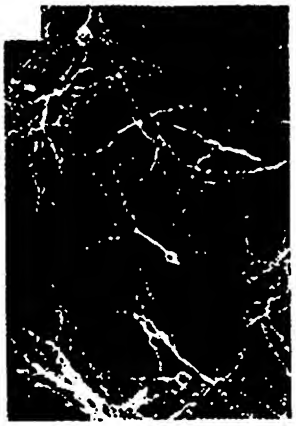


FIG.18B

FIG.18C

FIG.18D

FIG.18E

FIG.18F

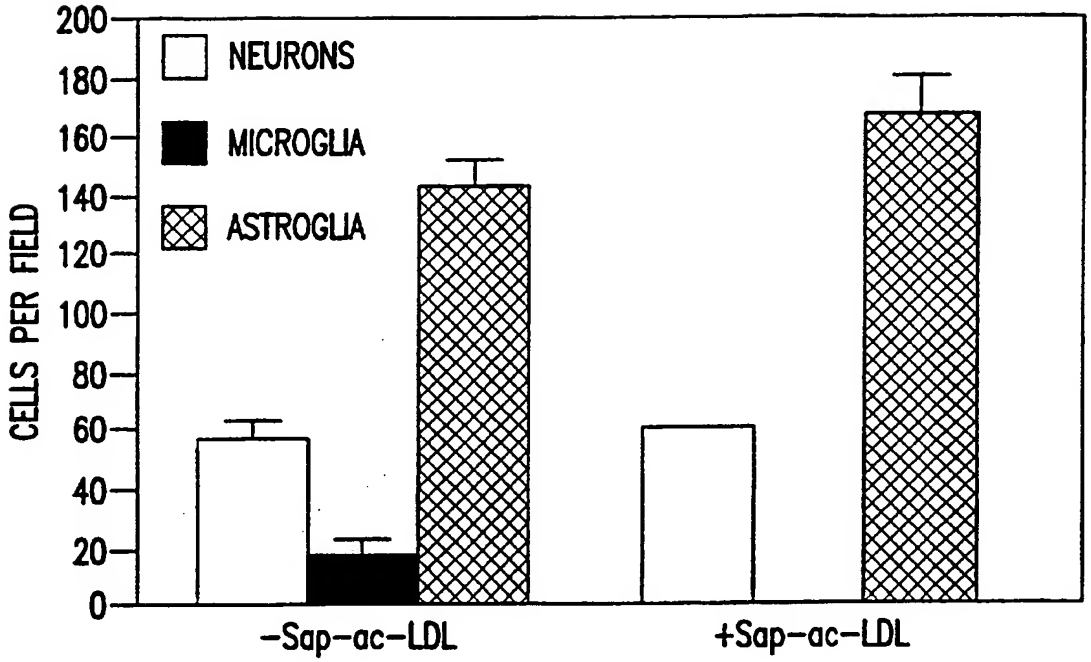

200
180
160
140
120
100
80
60
40
20
0
CELLS PER FIELD
NEURONS
MICROGLIA
ASTROGLIA
-Sap-ac-LDL
+Sap-ac-LDL

FIG.18G

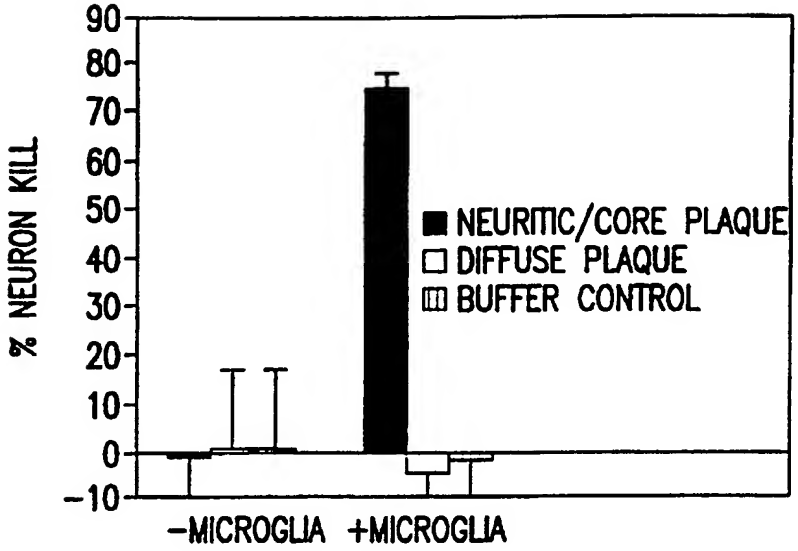


FIG.19A

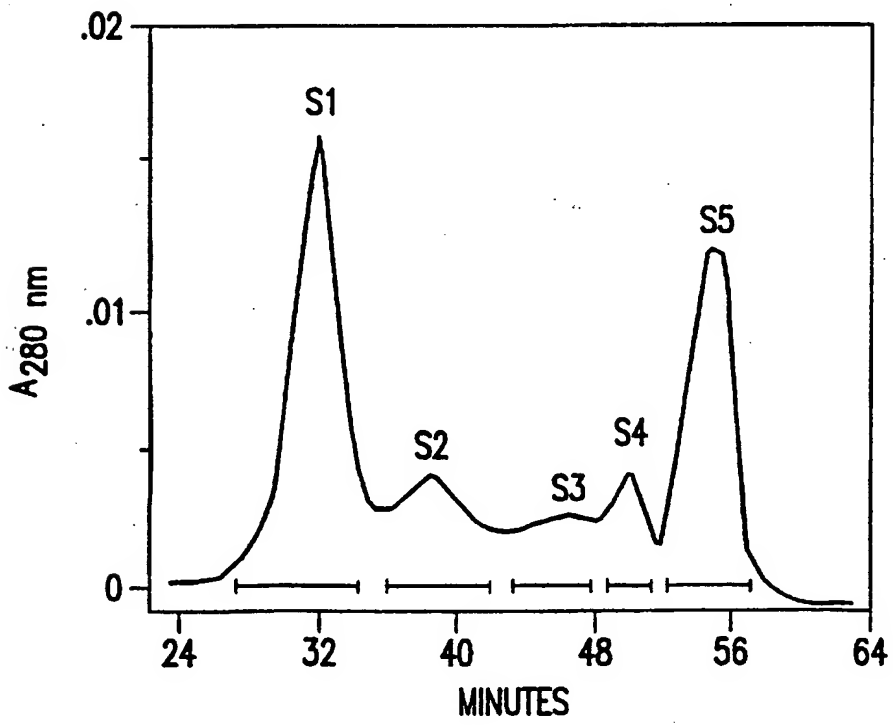


FIG.19B

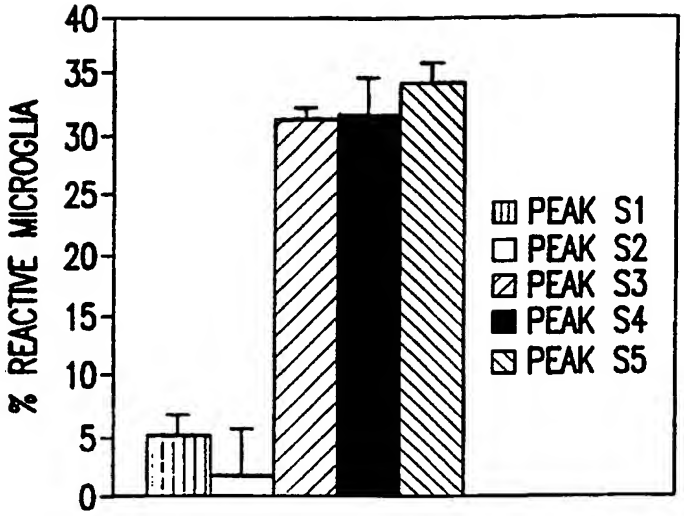


FIG.19C

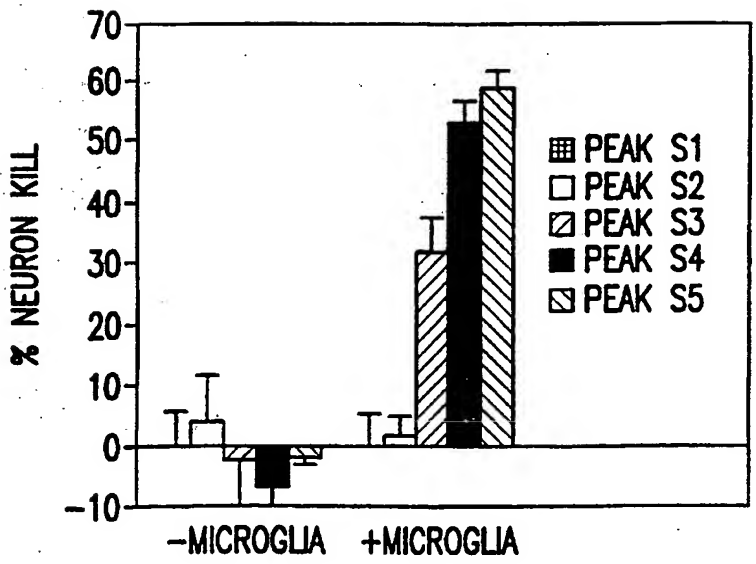


FIG.19D

FIG.20B

FIG.20D

FIG.20A

FIG.20C

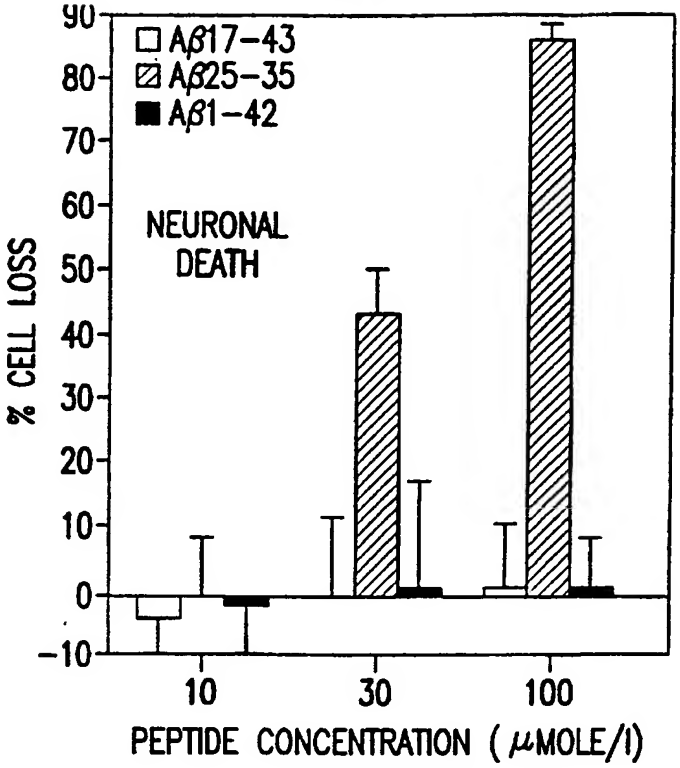


FIG.21A

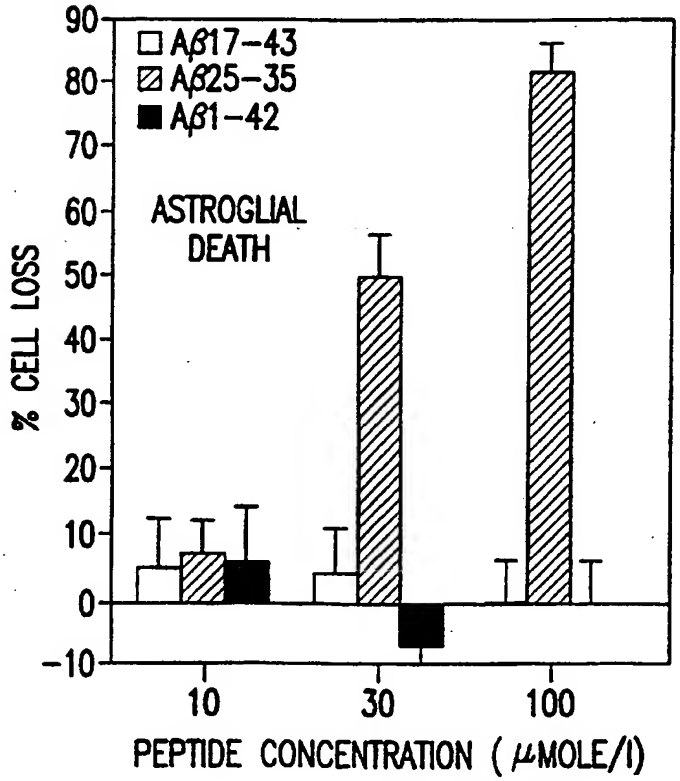


FIG.21B

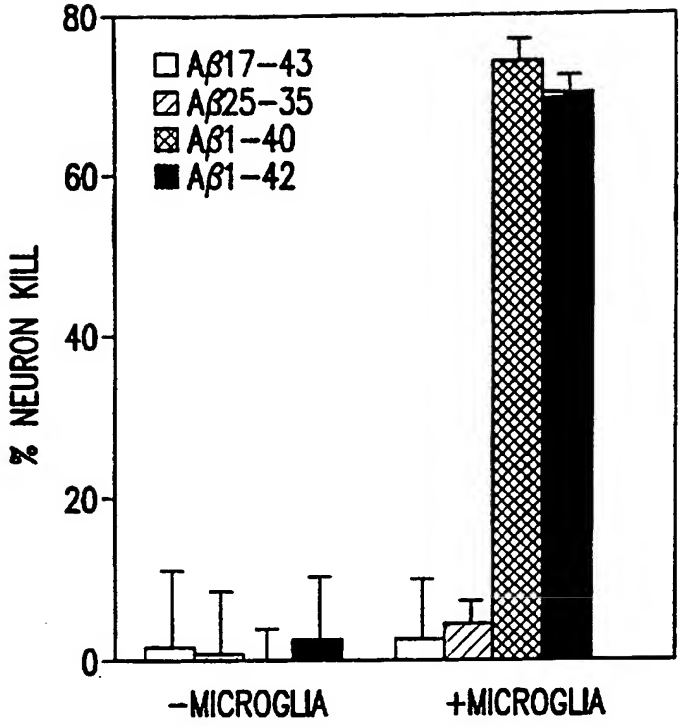


FIG.21C

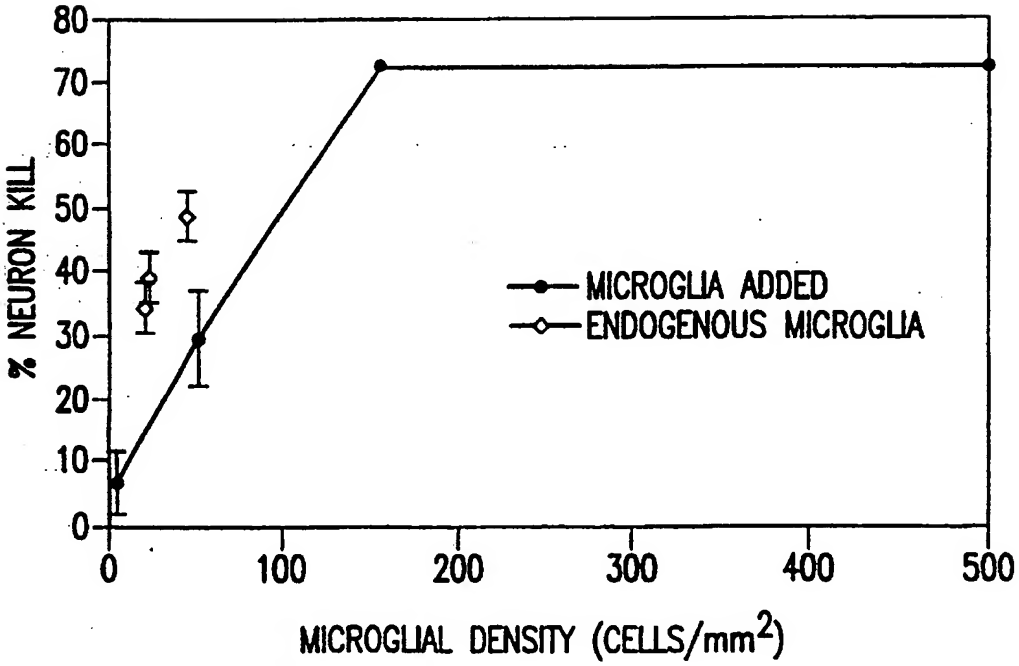


FIG.21D

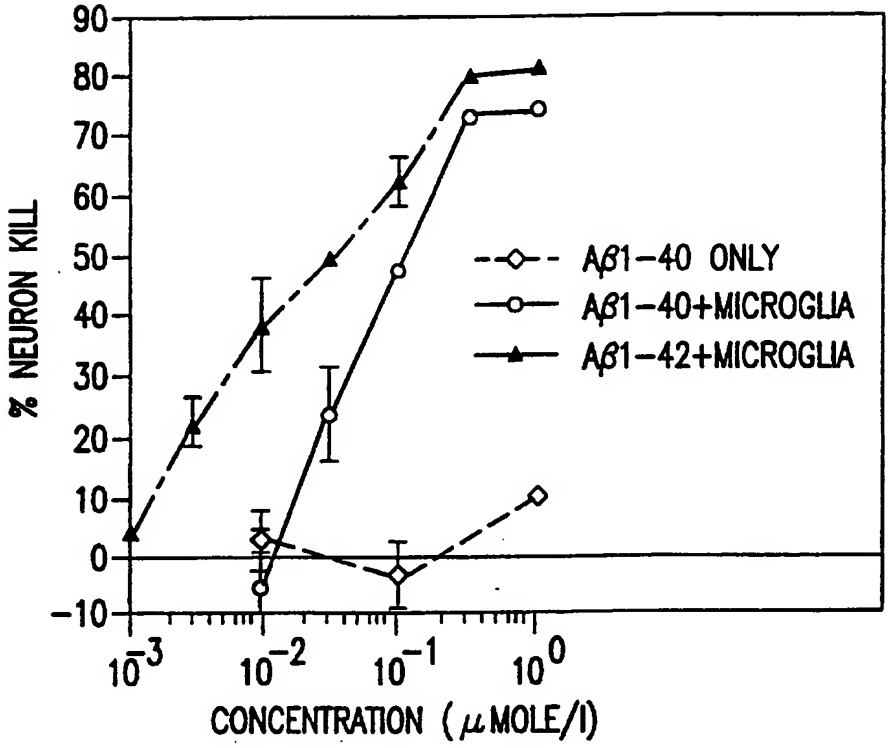

90
80
70
60
50
40
30
20
10
0
-10
% NEURON KILL
Aβ1-40 ONLY
Aβ1-40+MICROGLIA
Aβ1-42+MICROGLIA
10^{-3}
10^{-2}
10^{-1}
10^{0}
CONCENTRATION (μMOLE/l)

FIG.21E

FIG.22A

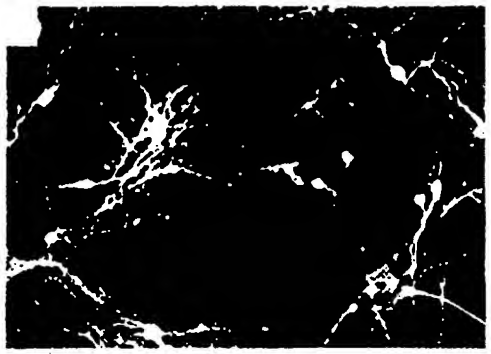


FIG.22B

FIG.22C

FIG.22D

FIG.22E

FIG.22F

FIG.23A

FIG.23B

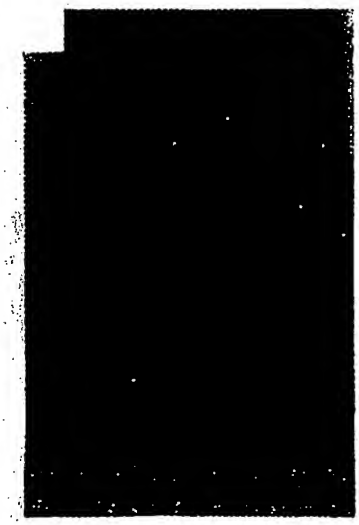


FIG.23C

FIG.23D

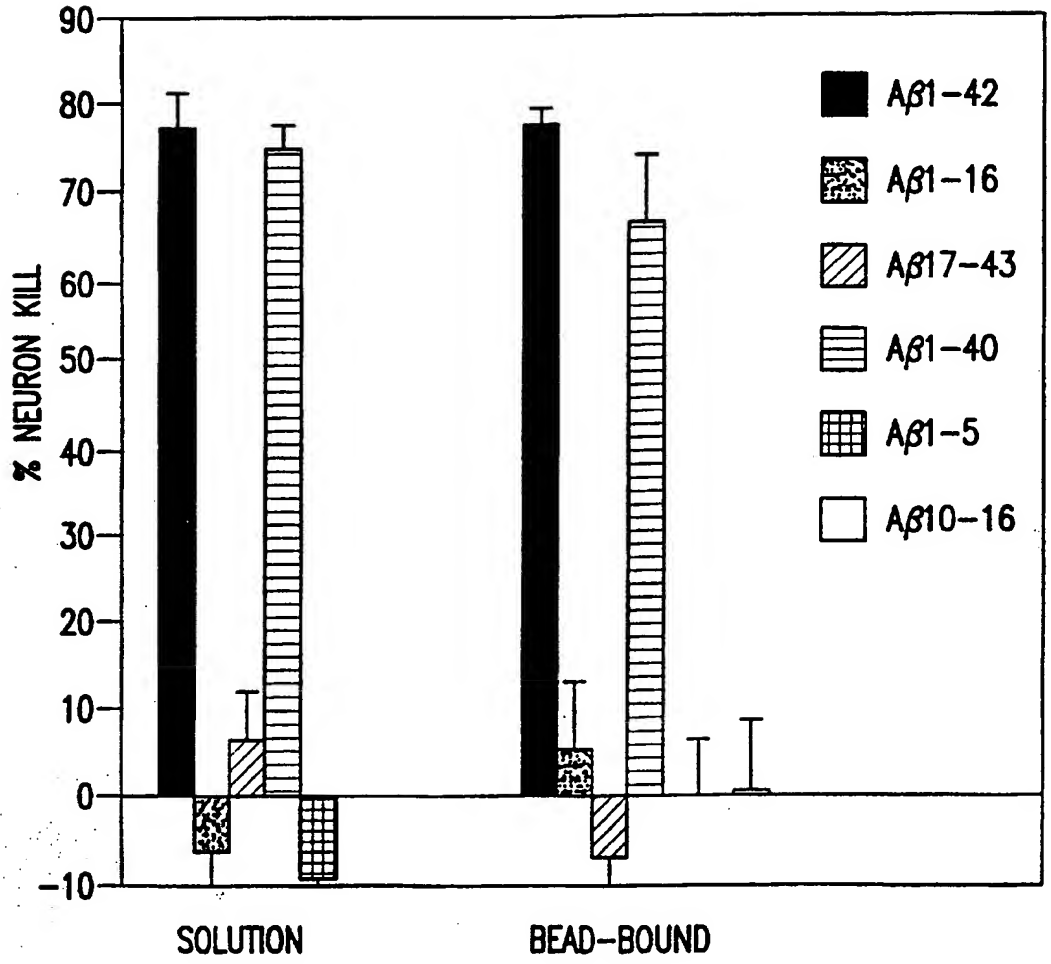

90
80
70
60
50
40
30
20
10
0
-10
% NEURON KILL
Aβ1-42
Aβ1-16
Aβ17-43
Aβ1-40
Aβ1-5
Aβ10-16
SOLUTION
BEAD-BOUND

FIG.23E

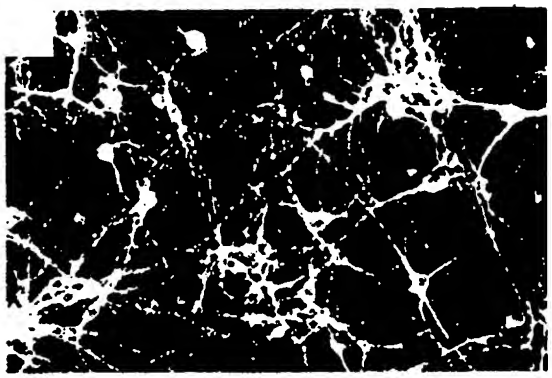


FIG.24A

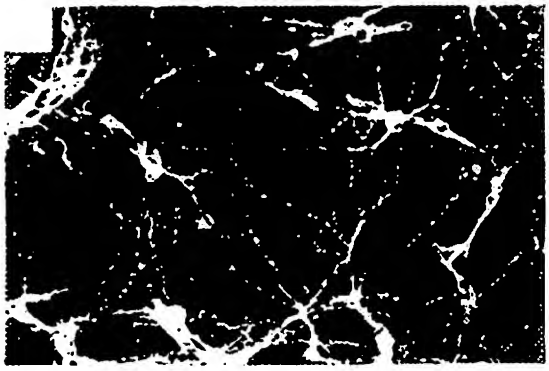


FIG.24B

FIG.24C

FIG.24D

FIG.24E

FIG.24F

FIG.24G

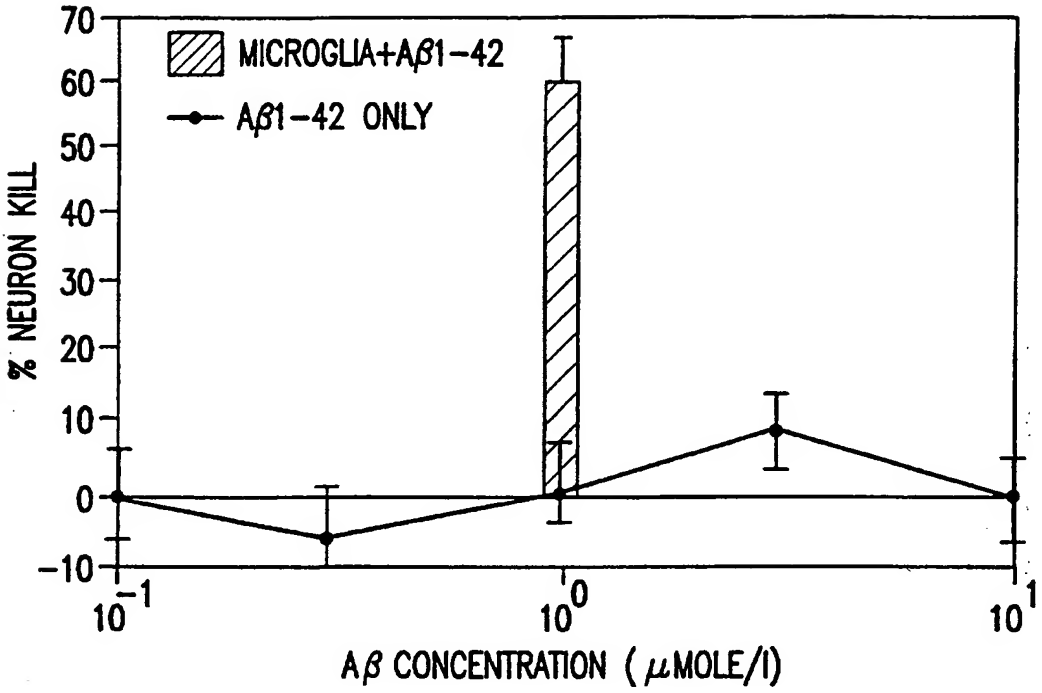

70
60
50
40
30
20
10
0
-10
% NEURON KILL
MICROGLIA+Aβ1-42
Aβ1-42 ONLY
10^-1
10^0
10^1
Aβ CONCENTRATION (μMOLE/l)

FIG.24H

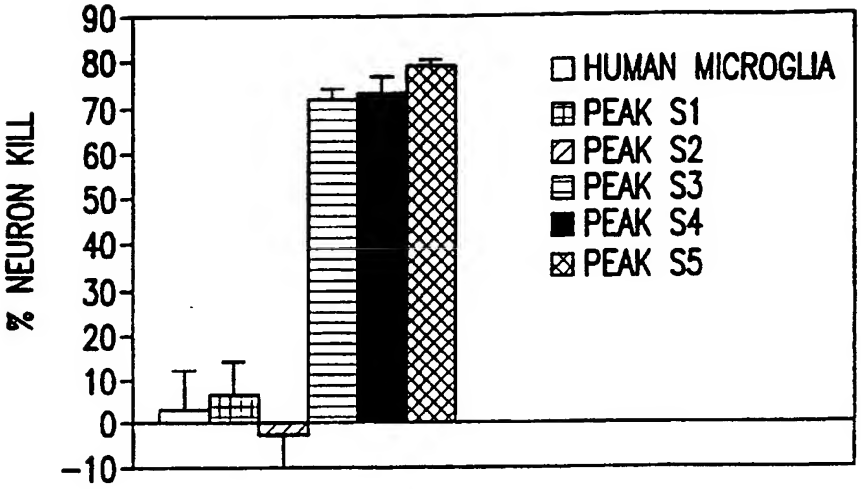


FIG.25A

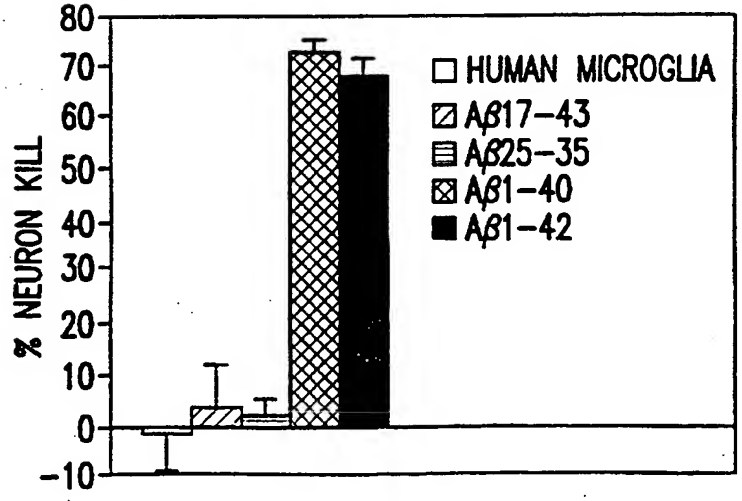


FIG.25B

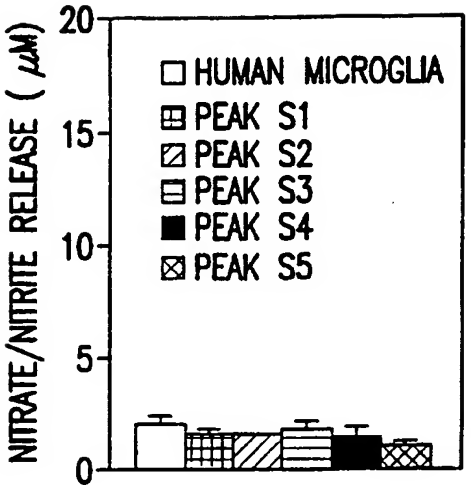


FIG.25C

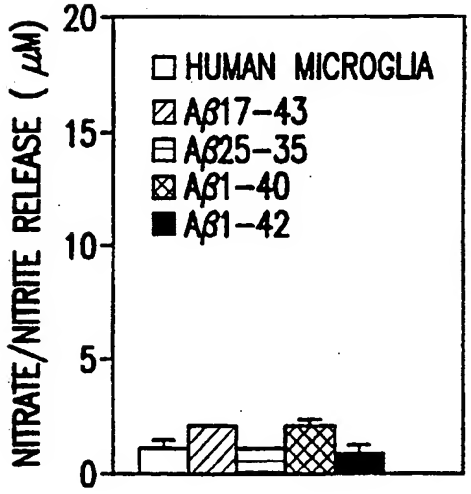


FIG.25D

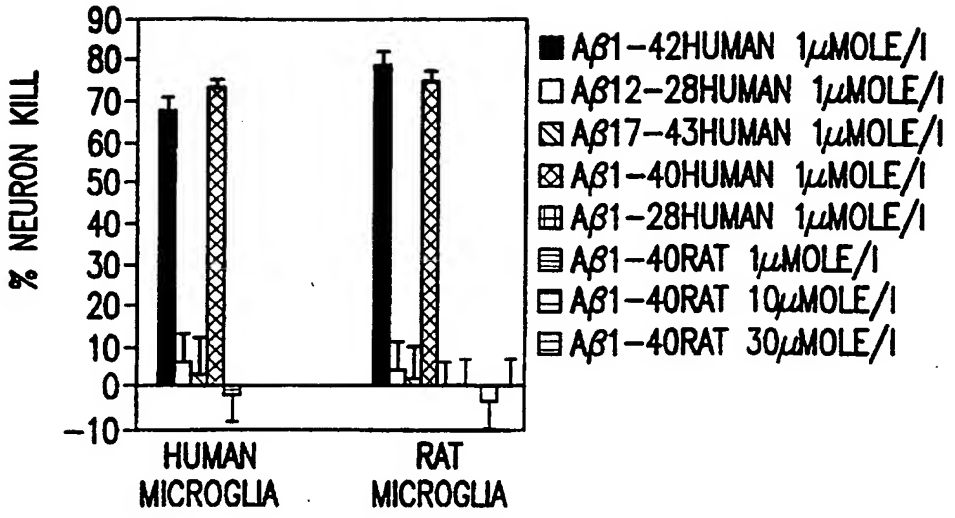

% NEURON KILL
90
80
70
60
50
40
30
20
10
0
−10
HUMAN MICROGLIA
RAT MICROGLIA
Aβ1−42HUMAN 1μMOLE/l
Aβ12−28HUMAN 1μMOLE/l
Aβ17−43HUMAN 1μMOLE/l
Aβ1−40HUMAN 1μMOLE/l
Aβ1−28HUMAN 1μMOLE/l
Aβ1−40RAT 1μMOLE/l
Aβ1−40RAT 10μMOLE/l
Aβ1−40RAT 30μMOLE/l

FIG.25E

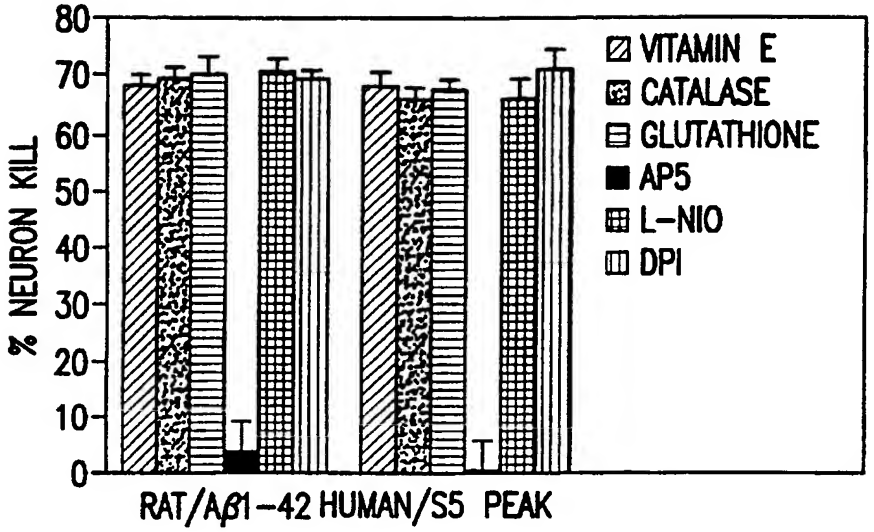


FIG.26A

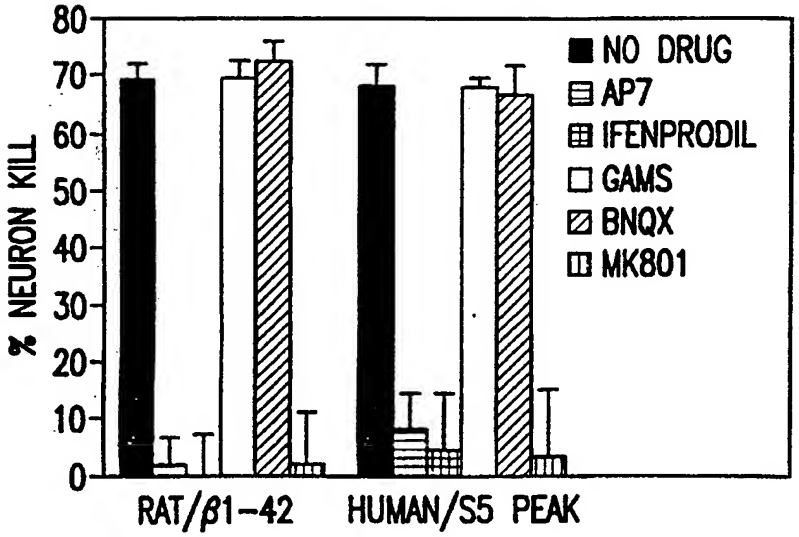


FIG.26B

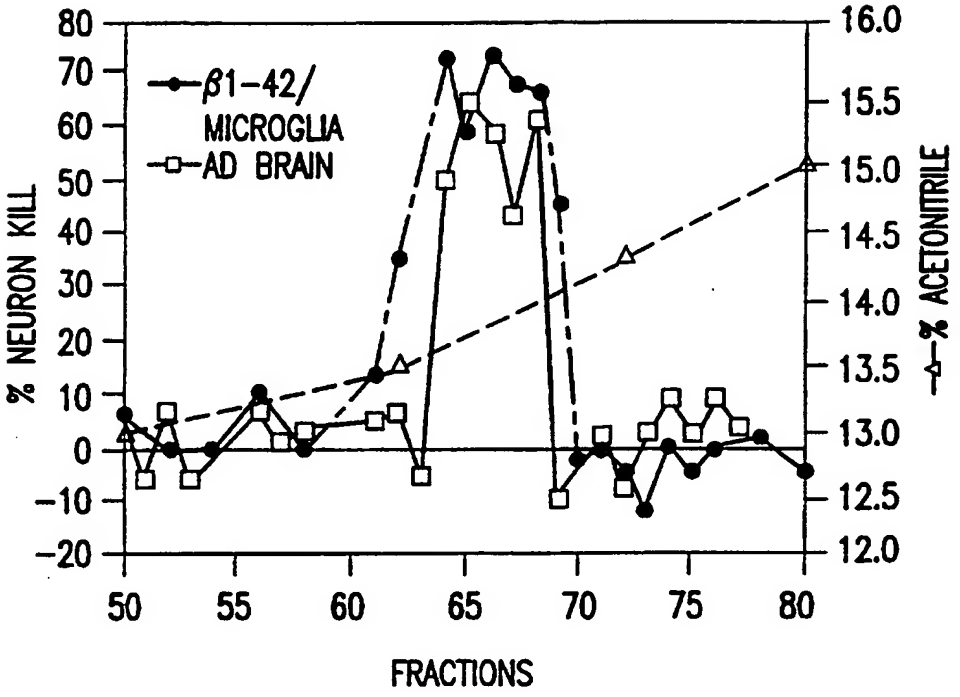

β1-42/
MICROGLIA
AD BRAIN
% NEURON KILL
% ACETONITRILE
FRACTIONS
80
70
60
50
40
30
20
10
0
-10
-20
16.0
15.5
15.0
14.5
14.0
13.5
13.0
12.5
12.0
50
55
60
65
70
75
80

FIG.26C

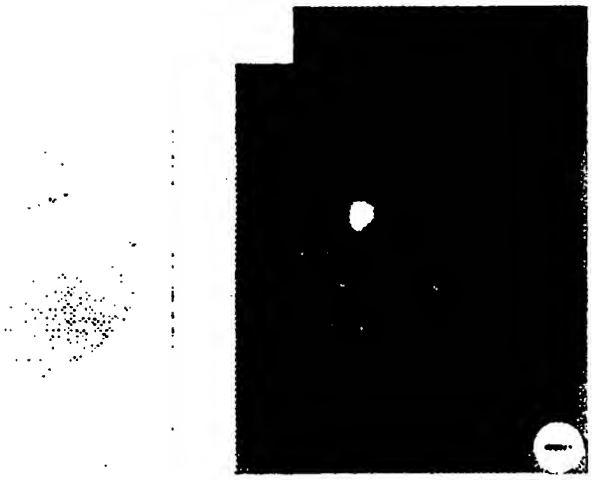


FIG.27A FIG.27B

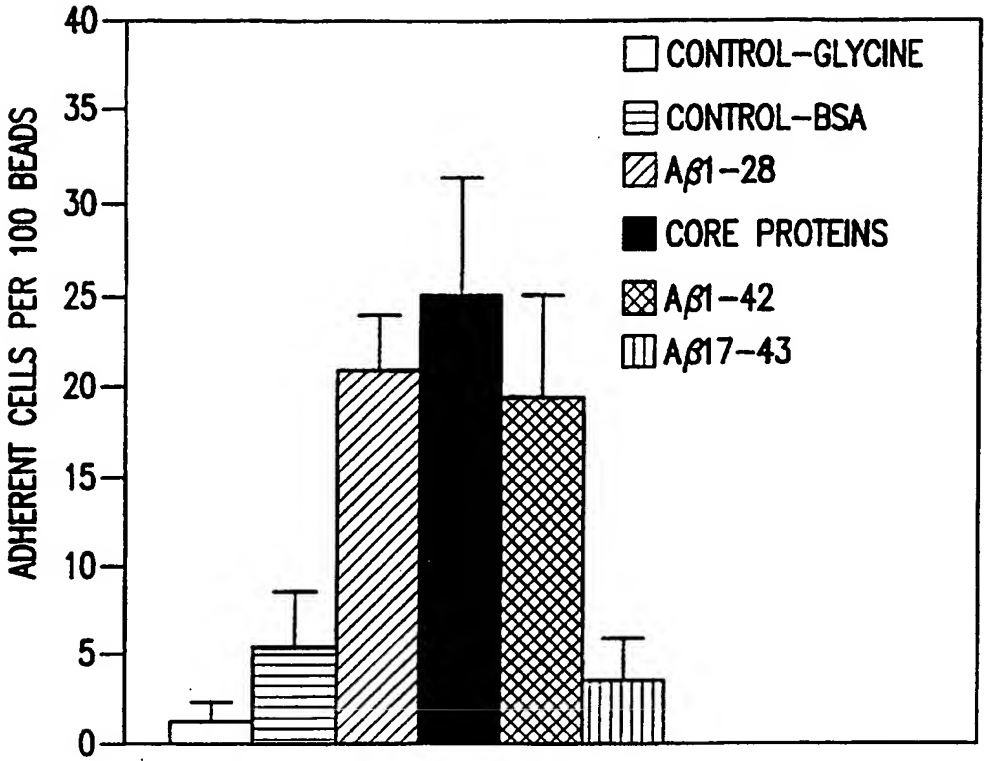

ADHERENT CELLS PER 100 BEADS
40
35
30
25
20
15
10
5
0
CONTROL–GLYCINE
CONTROL–BSA
Aβ1–28
CORE PROTEINS
Aβ1–42
Aβ17–43

FIG.27C

FIG.28A

FIG.28B

FIG.28C

FIG.28D

FIG.28E

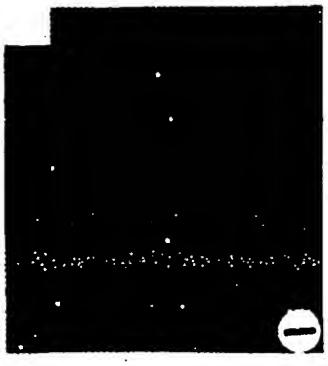


FIG.28F

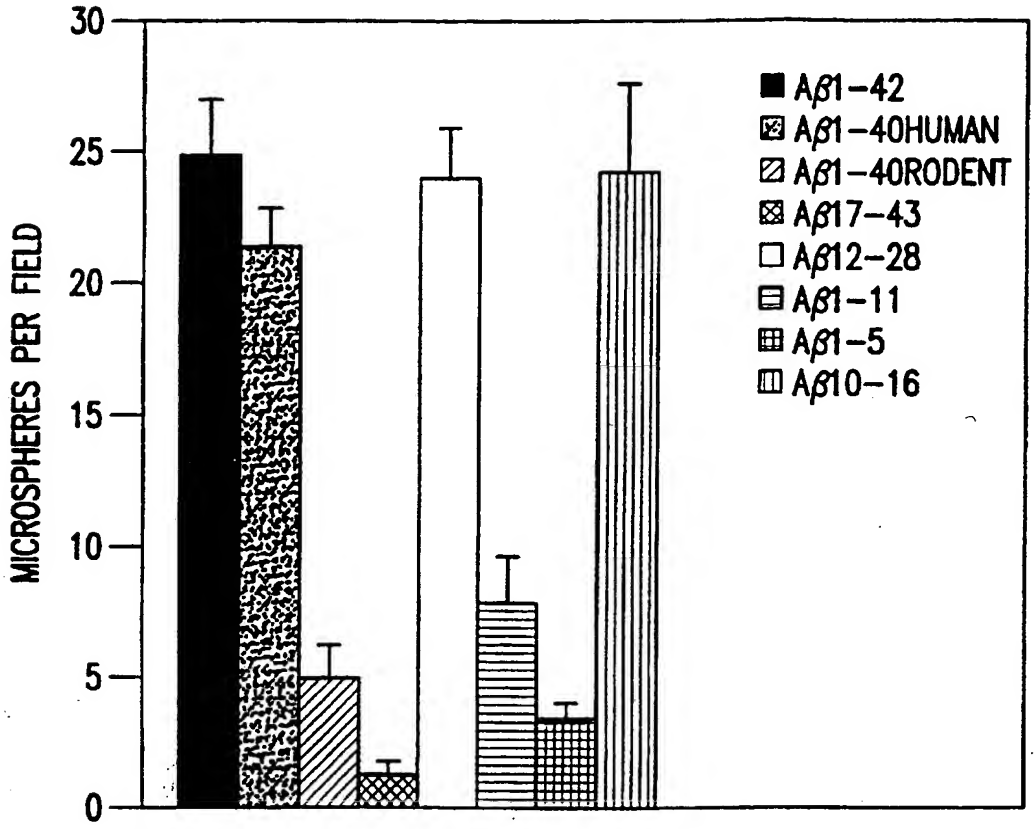

30
25
20
15
10
5
0
MICROSPHERES PER FIELD
Aβ1-42
Aβ1-40HUMAN
Aβ1-40RODENT
Aβ17-43
Aβ12-28
Aβ1-11
Aβ1-5
Aβ10-16

FIG.28G

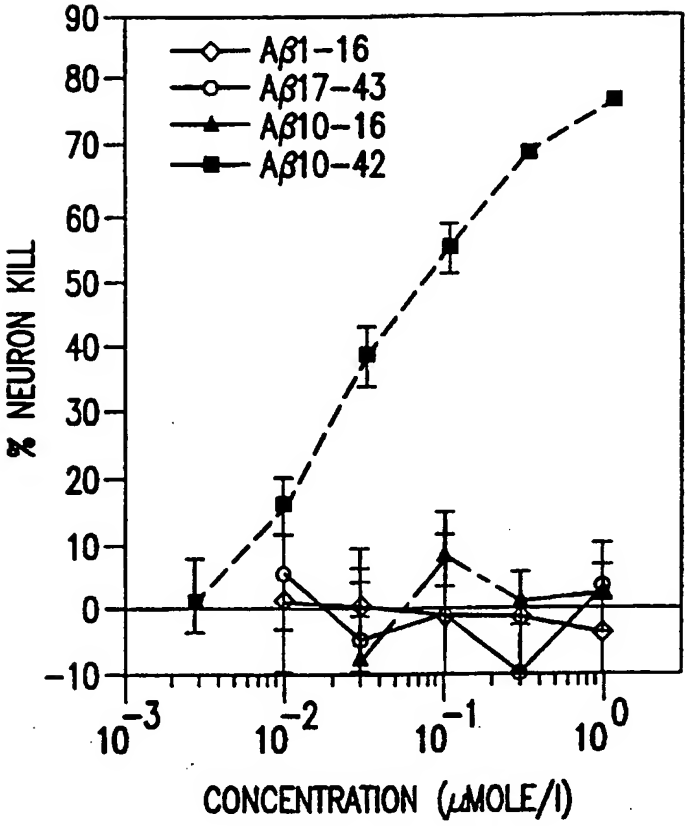

Aβ1-16
Aβ17-43
Aβ10-16
Aβ10-42
% NEURON KILL
90
80
70
60
50
40
30
20
10
0
-10
10^{-3}
10^{-2}
10^{-1}
10^{0}
CONCENTRATION (μMOLE/l)

FIG.29A

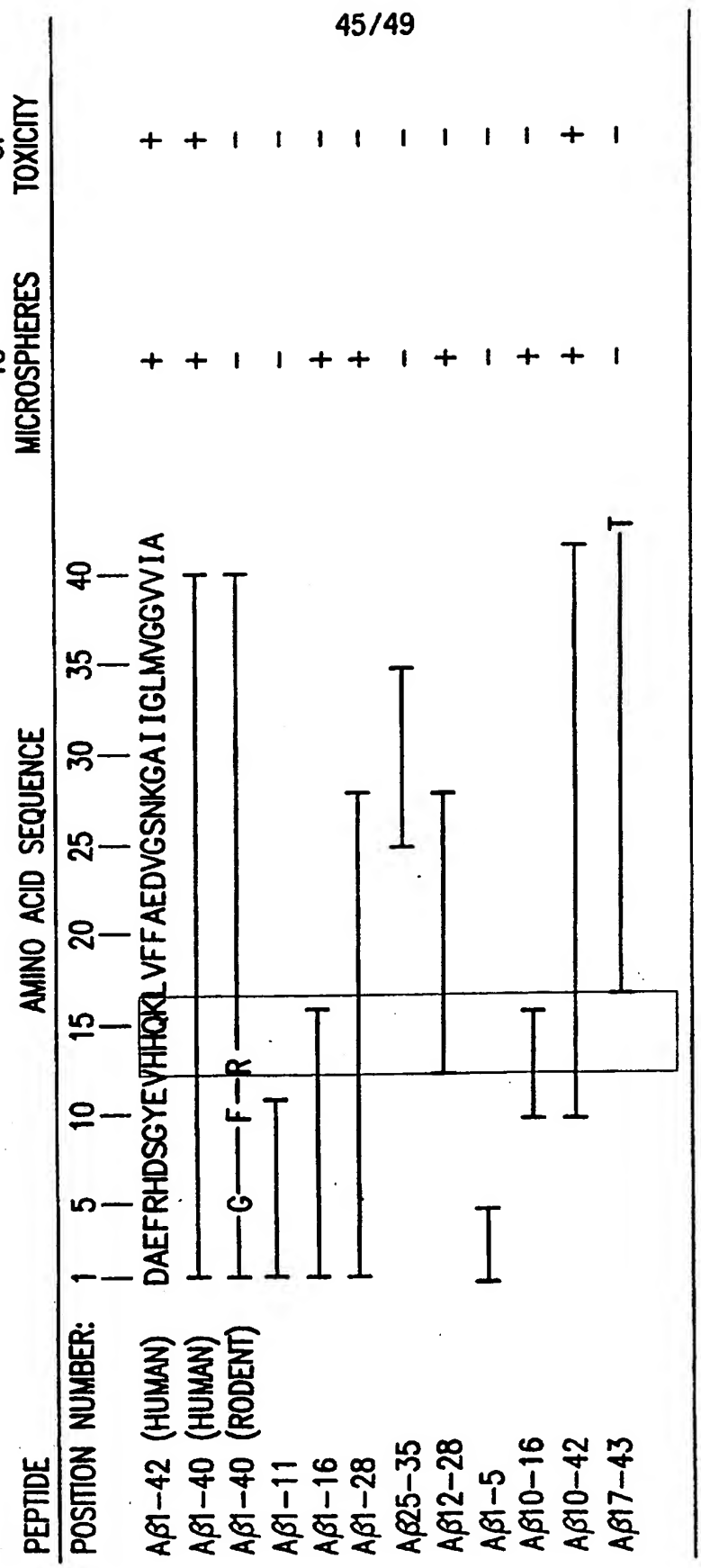

PEPTIDE
POSITION NUMBER:
AMINO ACID SEQUENCE
1 5 10 15 20 25 30 35 40
DAEFRHDSGYEVHHQKLVFFAEDVGSNKGAIIGLMVGGVVIA
Aβ1–42 (HUMAN)
Aβ1–40 (HUMAN)
Aβ1–40 (RODENT)
Aβ1–11
Aβ1–16
Aβ1–28
Aβ25–35
Aβ12–28
Aβ1–5
Aβ10–16
Aβ10–42
Aβ17–43
ADHERENCE TO MICROSPHERES
INDUCTION OF TOXICITY

FIG.29B

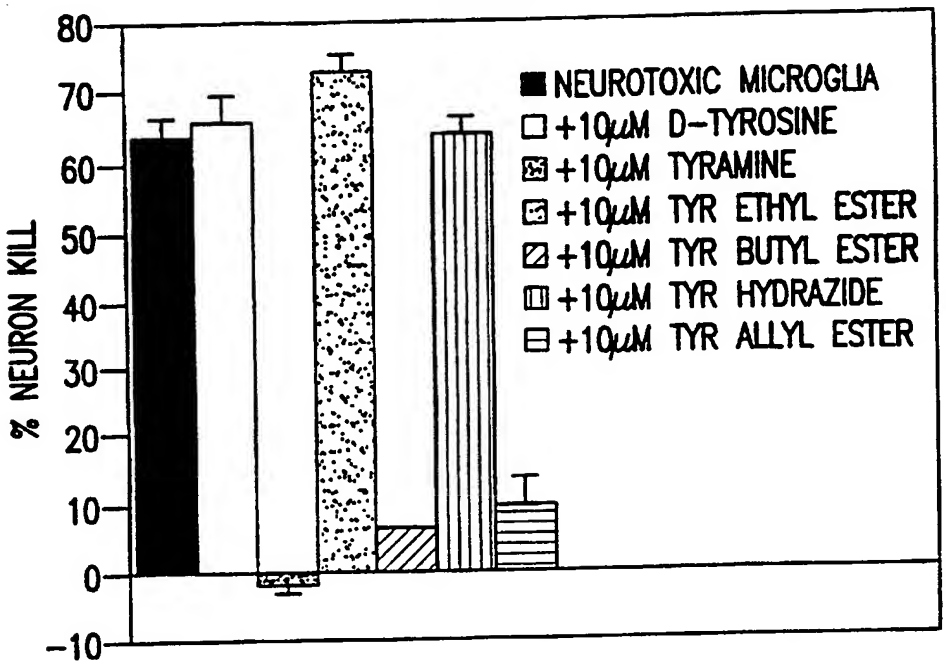

% NEURON KILL
80
70
60
50
40
30
20
10
0
-10
NEUROTOXIC MICROGLIA
+10μM D-TYROSINE
+10μM TYRAMINE
+10μM TYR ETHYL ESTER
+10μM TYR BUTYL ESTER
+10μM TYR HYDRAZIDE
+10μM TYR ALLYL ESTER

FIG.30

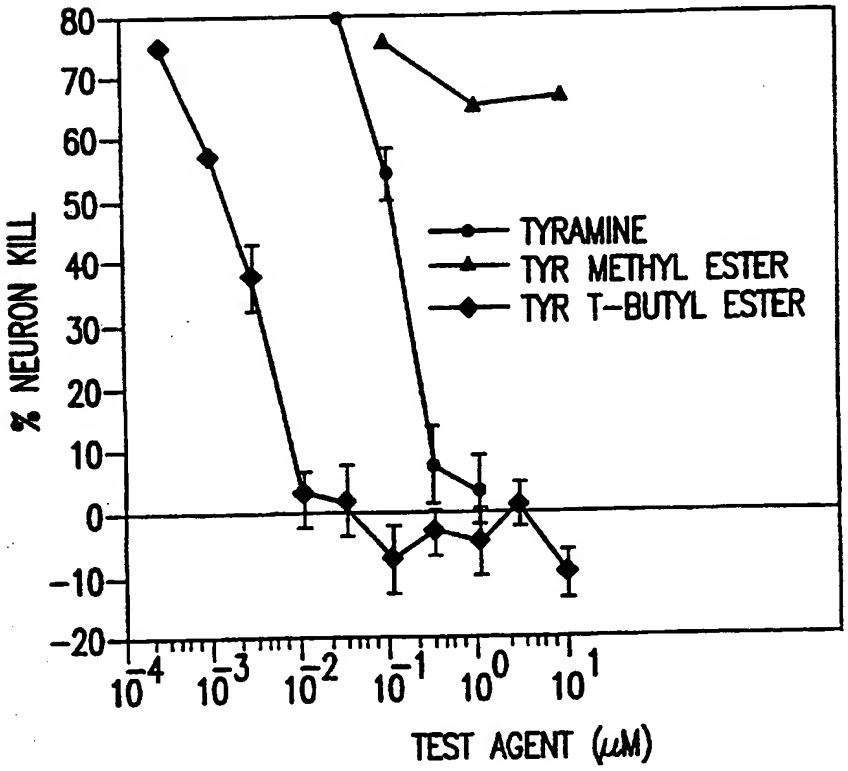

% NEURON KILL
80
70
60
50
40
30
20
10
0
-10
-20
TYRAMINE
TYR METHYL ESTER
TYR T-BUTYL ESTER
10^{-4} 10^{-3} 10^{-2} 10^{-1} 10^{0} 10^{1}
TEST AGENT (μM)

FIG.31

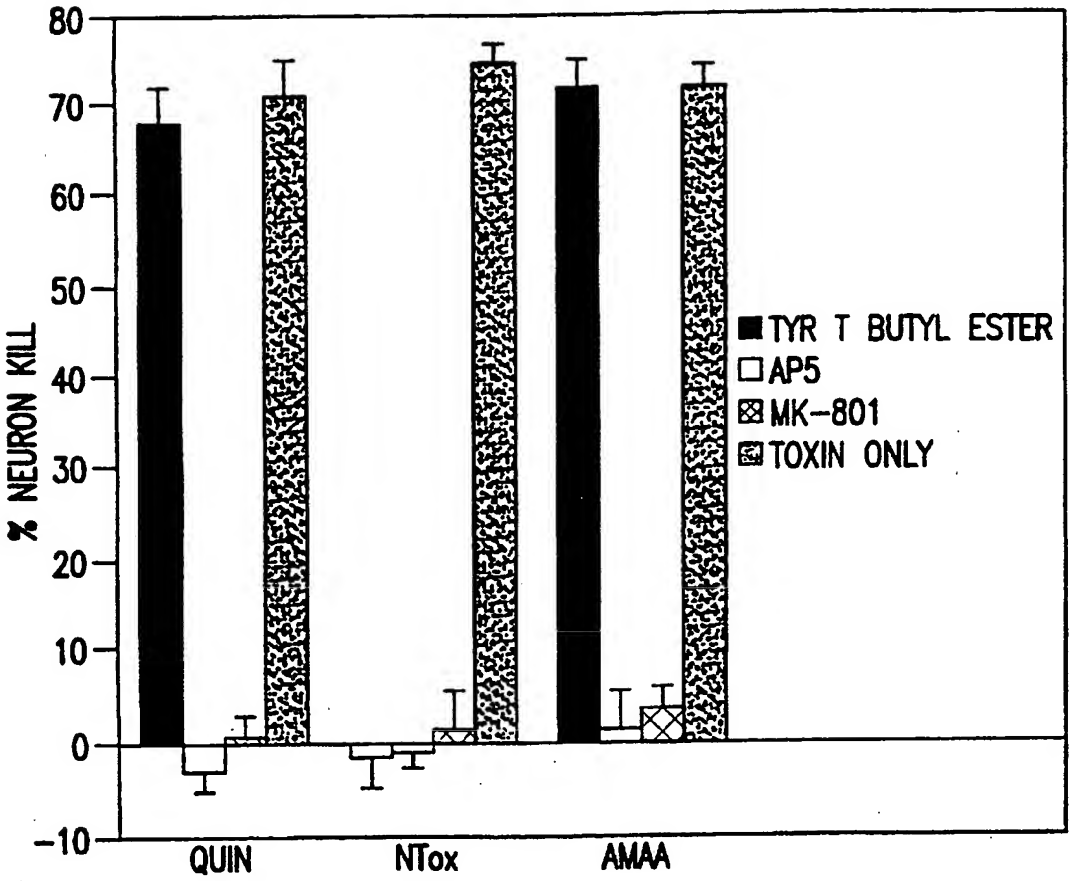
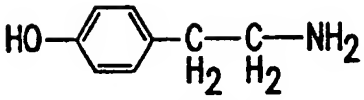
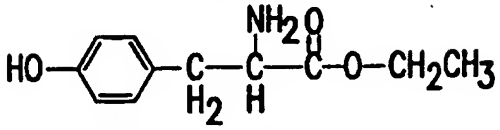

80
70
60
50
40
30
20
10
0
-10
% NEURON KILL
QUIN
NTox
AMAA
TYR T BUTYL ESTER
AP5
MK-801
TOXIN ONLY

FIG.32



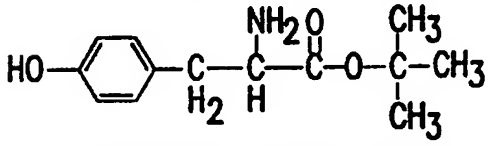
⊕

TYRAMINE



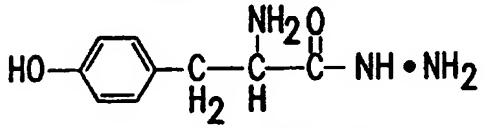
⊖

L-TYROSINE ETHYL ESTER



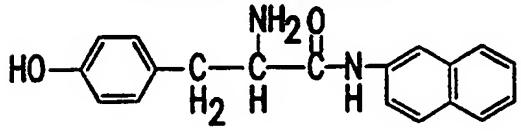
(++)

L-TYROSINE t-BUTYL ESTER



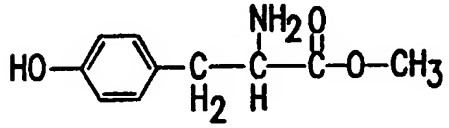
⊖

L-TYROSINE HYDRAZIDE



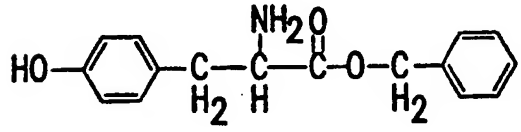
(++)

L-TYROSINE 2-NAPHTHYLAMIDE



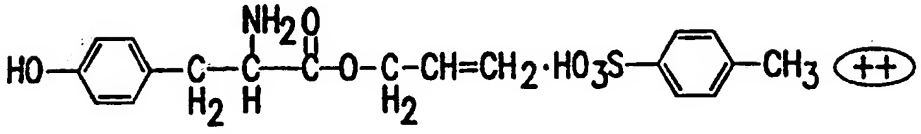
⊖

DL-TYROSINE METHYL ESTER



(++)

L-TYROSINE BENZYL ESTER



(++)

L-TYROSINE ALLYL ESTER p-TOLUENESULFONATE

FIG.33

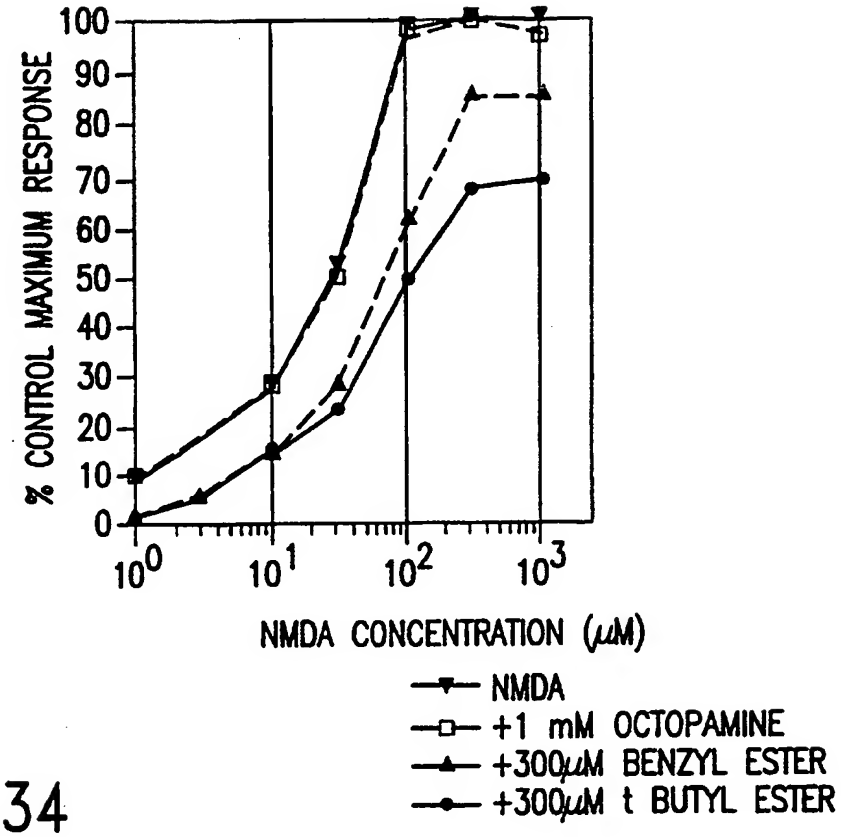

% CONTROL MAXIMUM RESPONSE
100
90
80
70
60
50
40
30
20
10
0
10^0
10^1
10^2
10^3
NMDA CONCENTRATION (μM)
NMDA
+1 mM OCTOPAMINE
+300μM BENZYL ESTER
+300μM t BUTYL ESTER

FIG.34

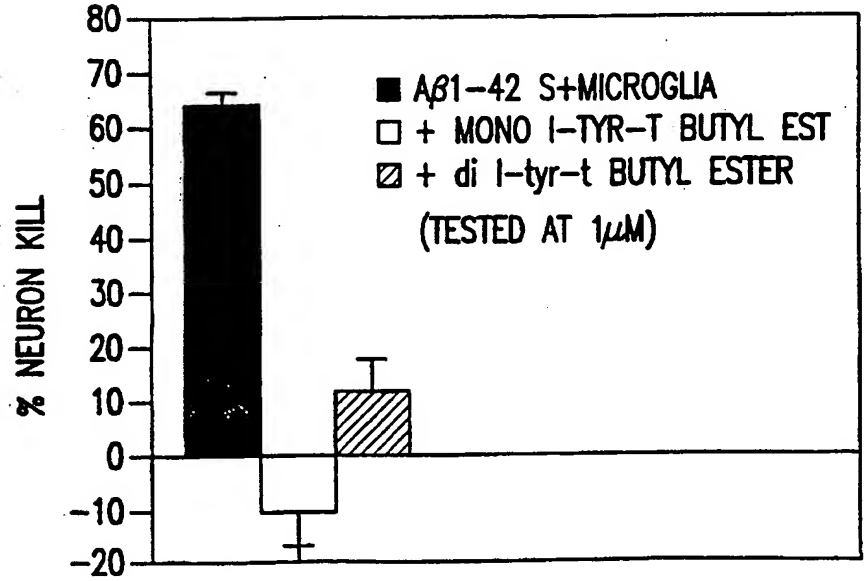

% NEURON KILL
80
70
60
50
40
30
20
10
0
-10
-20
Aβ1-42 S+MICROGLIA
+ MONO l-TYR-T BUTYL EST
+ di l-tyr-t BUTYL ESTER
(TESTED AT 1μM)

FIG.35

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US97/16999

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :A61K 49/00, G01N 31/00, 33/48, 33/53, 33/567, 33/569
US CL :424/9.1, 9.29.32, 9.321, 9.322, 9.341; 435/6, 7.1, 7.2, 7.21, 7.8

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/9.1, 9.29.32, 9.321, 9.322, 9.341; 435/6, 7.1, 7.2, 7.21, 7.8

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	SIRIPONT et al. Receptor-mediated binding of the acute-phase reactant mouse serum amyloid P-component (SAP) to macrophages. Cellular Immunology. 1988. Vol. 117, No. 2, pages 239-252, see entire document.	1-2, 5, 9-10, 16
A	EIKELENBOOM et al. Cerebral amyloid plaques in Alzheimer's disease but not in scrapie-affected mice are closely associated with a local inflammatory process. Virchows Archiv. 1991. Vol. 60, No. 5, pages 329-339, see entire document.	1-23

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"B" earlier document published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
30 DECEMBER 1997	30 JAN 1998
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231	Authorized officer PATRICIA A. DUFFY
Facsimile No. (703) 305-3230	Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US97/16999

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	MEDA et al. ß-Amyloid (25-35) peptide and IFN-gamma synergistically induce the production of the chemotactic cytokine MCP-1/JE in monocytes and microglial cells. Journal of Immunology. 1996. Vol. 157, pages 1213-1218, see entire document.	1-23

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US97/16999

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:
1-23

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US97/16999

B. FIELDS SEARCHED
Electronic data bases consulted (Name of data base and where practicable terms used):

APS, Dialog, Medline, CAB Abstracts, EMBASE, JAPIO, Derwent WPI, Derwent Biotechnology Abstracts, Biosis . search terms: monocyte, glial, microglial, microglia, plaque, amyloid, apolipoprotein E, beta amyloid, Alzheimer's, Lewy Body, Parkinson's, dementia, phagocyte, neuron, neuronal, toxic, toxicity, activation, activate, bind, complex, receptor(s), screening, assay, inhibitor, modulate, solid phase, liposome, implantation, image, imaging, detection.

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING
This ISA found multiple inventions as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single inventive concept under PCT Rule 13.1. In order for all inventions to be searched, the appropriate additional search fees must be paid.

Group I, claim(s) 1-23, drawn to screening methods to identify compounds which inhibit complex formation between plaque components and monocuclear phagocytes.

Group II, claim(s) 24-46, drawn to screening methods to identify copounds which inhibit activation of mononuclear phaocytes by a plaque component.

Group III, claim(s) 47-69, drawn to screening methods to identify components which inhibit plaque component neurotoxicity of a mononuclear phagocyte.

Group IV, claim(s)70-90, drawn to screening methods to identify compounds which inhibit the effect of a neurotoxin from a plaque component activated mononuclear phagocyte on a neuron.

Group V, claim(s) 91-93, drawn to HHQK-like compounds.

Group VI, claim(s) 94-96, drawn to chloroquine compounds.

Group VII, claim(s)97-99 and 142, drawn to tyramine compounds.

Group VIII, claim(s) 100-136, drawn to methods of treatment of neurologic disease using tyramine compounds.

Group IX, claim(s) 137-140, drawn to screening methods to identify compounds which inhibit neurotoxic activity of neurotoxic compounds on neurons.

Group X, claim 141, drawn to methods of assaying for a neurotoxin *in vivo* in a patient.

The inventions listed as Groups I-X do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons:

The special technical feature of Group I-IV, IX and X are plaque components. The plaque components of neurologic disease are known in the prior art. It is well established in the art that *b*-amyloid, heparin sulfate proteoglycans, apolipoproteins, and lysosomal enzymes were present in amyloid forming plaques in amyloid-forming diseases such as Alzhiemer's disease. The toxicity of the plaque components such as *b*-amyloid was also established in the art at the time the disclosure was filed. Thus, pursuant to PCT Rule 13.1 the special technical feature of the methods does not define a novel contribution over the prior art. Thus, the methods employing the special technical feature of the plaque components are deemed to lack unity of invention.

The special technical feature of Group V is HHQK-like compounds, the special technical feature of Group VI is tyramine compounds, and the special technical feature of Groups VII and VIII are the tyramine compounds. The special technical features are deemed distinct from the special technical feature of Groups I-IV, IX and X and from each other because they have different chemical structures, have different functions and are made by different methods. In addition, Group VI tyramine compounds and Group VII chloroquine compounds are publically available and are known in the prior art. Thus, the special technical features do not relate to a single inventive concept and do not define a novel contribution over the prior art compounds and thus the special technical features as defined by the chemical compounds and methods employing the special technical features of these chemical compounds are deemed to lack unity of invention. Therefore, the claims are not so linked by a special technical feature within the meaning of PCT Rule 13.2 so as to form a single inventive concept.